

# Expanding the Chemical Toolbox for the Investigation of $\text{Ap}_3\text{A}$

## Dissertation

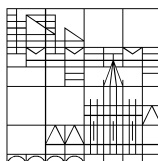
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# Abbreviations

|                   |                                                                                                         |
|-------------------|---------------------------------------------------------------------------------------------------------|
| 2D                | two-dimensional                                                                                         |
| μg                | microgram                                                                                               |
| μL                | microliter                                                                                              |
| μM                | micromolar                                                                                              |
| μmol              | micromole                                                                                               |
| Å                 | angstrom                                                                                                |
| a.u.              | atomic units                                                                                            |
| aaRS              | aminoacyl-tRNA synthetase                                                                               |
| ABP               | affinity-based probe                                                                                    |
| ABPP              | affinity-based proteomic profiling                                                                      |
| ADP               | adenosine 5'-diphosphate                                                                                |
| AMP               | adenosine 5'-monophosphate                                                                              |
| AP                | alkaline phosphatase                                                                                    |
| Ap <sub>3</sub> A | diadenosine triphosphate                                                                                |
| Ap <sub>4</sub> A | diadenosine tetraphosphate                                                                              |
| Ap <sub>n</sub> A | diadenosine polyphosphate                                                                               |
| APS               | ammonium peroxodisulfate                                                                                |
| ArgRS             | arginyl-tRNA synthetase                                                                                 |
| ARTD <sub>1</sub> | ADP-ribosyltransferase diphtheria toxin-like 1                                                          |
| ATP               | adenosine 5'-triphosphate                                                                               |
| BCIP              | 5-bromo-4-chloro-3-indolyl phosphate                                                                    |
| BSA               | bovine serum albumin                                                                                    |
| BTAA              | 2-(4-((bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid |
| <i>c</i>          | concentration                                                                                           |
| CBS               | cystathionine beta synthase                                                                             |
| CDI               | <i>N,N'</i> -carbonyldiimidazole                                                                        |
| CFS               | common fragile site                                                                                     |
| CID               | collision-induced dissociation                                                                          |
| CNS               | central nervous system                                                                                  |
| CoA               | coenzyme A                                                                                              |
| CuAAC             | copper(I)-catalyzed azide-alkyne cycloaddition                                                          |
| d                 | doublet                                                                                                 |
| DCC               | <i>N,N'</i> -dicyclohexylcarbodiimide                                                                   |
| DCI               | 4,5-dicyanoimidazole                                                                                    |
| DCM               | dichloromethane                                                                                         |
| dd                | doublet of doublet                                                                                      |
| DEAE              | diethylaminoethanol                                                                                     |
| DMEM              | Dulbecco's modified eagle medium                                                                        |
| DMF               | dimethylformamide                                                                                       |

|                         |                                                    |
|-------------------------|----------------------------------------------------|
| DMSO                    | dimethyl sulfoxide                                 |
| DNA                     | deoxyribonucleic acid                              |
| dNTP                    | deoxyribonucleotide triphosphate                   |
| DSB                     | double-strand breaks                               |
| dt                      | doublet of triplet                                 |
| DTT                     | dithiothreitol                                     |
| dTTP                    | deoxythymidine triphosphate                        |
| <i>E</i>                | FRET efficiency                                    |
| <i>E. coli</i>          | <i>Escherichia coli</i>                            |
| e.g.                    | exempli gratia                                     |
| EDC                     | 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide      |
| EDTA                    | ethylenediamine tetraacetate                       |
| EGFR                    | epidermal growth factor receptor                   |
| eq.                     | equivalent                                         |
| ESI                     | electrospray ionization                            |
| ETT                     | 5-(ethylthio)-1 <i>H</i> -tetrazole                |
| FDR                     | false discovery rate                               |
| Fdxr                    | ferredoxin reductase                               |
| Fhit                    | fragile histidine triad protein                    |
| <i>FI</i>               | fluorescence intensity                             |
| FRET                    | Förster resonance energy transfer                  |
| g                       | gram                                               |
| GlnRS                   | glutaminyl-tRNA synthetase                         |
| GO                      | <i>Gene Ontology</i>                               |
| GST                     | glutathione- <i>S</i> -transferase                 |
| h                       | hour                                               |
| HCD                     | higher-energy collisional dissociation             |
| HEK                     | human embryonic kidney                             |
| HEPES                   | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| Hint1                   | histidine triad nucleotide-binding protein 1       |
| His                     | histidine                                          |
| HisRS                   | histidine tRNA synthetase                          |
| Hit                     | histidine triad                                    |
| HPLC                    | high performance liquid chromatography             |
| HR                      | high resolution                                    |
| HRP                     | horse-radish peroxidase                            |
| Hsp60                   | 60 kDa heat shock protein                          |
| Hz                      | hertz                                              |
| <i>IC</i> <sub>50</sub> | half maximal inhibitory concentration              |
| IgE-Ag                  | immunoglobulin E-antigen                           |
| IMP                     | inosine 5'-monophosphate                           |
| IPTG                    | isopropyl $\beta$ -D-1-thiogalactopyranoside       |
| IR                      | infrared                                           |

|                              |                                                              |
|------------------------------|--------------------------------------------------------------|
| <i>J</i>                     | coupling constant                                            |
| K <sub>ATP</sub> channel     | ATP-sensitive potassium channel                              |
| <i>K<sub>D</sub></i>         | dissociation constant                                        |
| kDa                          | kilodalton                                                   |
| <i>k<sub>obs</sub></i>       | observed rate constant                                       |
| LC-MS/MS                     | liquid chromatography tandem mass spectrometry               |
| LFQ                          | label free quantification                                    |
| log                          | logarithm                                                    |
| LysRS                        | lysine-tRNA synthetase                                       |
| LysU                         | heat inducible lysine-tRNA synthetase                        |
| M                            | molar                                                        |
| m                            | multiplet                                                    |
| <i>m/z</i>                   | mass to charge ratio                                         |
| mA                           | milliampere                                                  |
| MAPK                         | mitogen-activated protein kinase                             |
| mg                           | milligram                                                    |
| MHz                          | megahertz                                                    |
| min                          | minute                                                       |
| MITF                         | microphthalmia transcription factor                          |
| mL                           | milliliter                                                   |
| mM                           | milimolar                                                    |
| mm                           | millimeter                                                   |
| mmol                         | millimol                                                     |
| MPLC                         | medium pressure liquid chromatography                        |
| MQ                           | milliQ water                                                 |
| MS                           | mass spectrometry                                            |
| MTT                          | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MW                           | microwave or molecular weight                                |
| MWCO                         | molecular weight cut-off                                     |
| NAD <sup>+</sup>             | nicotinamide adenine dinucleotide                            |
| NHS                          | <i>N</i> -hydroxysuccinimide                                 |
| NitI                         | nitrilase I                                                  |
| nm                           | nanometer                                                    |
| nM                           | nanomolar                                                    |
| NMI                          | <i>N</i> -methylimidazole                                    |
| NMR                          | nuclear magnetic resonance                                   |
| N <sub>p<sub>n</sub></sub> N | dinucleoside polyphosphate                                   |
| NTA                          | nitrilotriacetic acid                                        |
| nudix                        | nucleoside diphosphate-linked moiety X                       |
| NudT2                        | nudix motif 2                                                |
| OD <sub>600</sub>            | optical density at 600 nm                                    |
| on                           | overnight                                                    |
| p.a.                         | per analysis                                                 |
| PBS                          | phosphate buffered saline                                    |

## Abbreviations

---

|          |                                                           |
|----------|-----------------------------------------------------------|
| PEBPI    | phosphatidylethanolamine-binding protein 1                |
| ppm      | parts per million                                         |
| PSM      | peptide spectrum match                                    |
| PTM      | post-translational modification                           |
| q        | quartet                                                   |
| $R$      | distance                                                  |
| $R_0$    | Förster distance                                          |
| $R_f$    | retention factor                                          |
| ROS      | reactive oxygen species                                   |
| RP       | reversed-phase                                            |
| rpm      | rounds per minute                                         |
| rt       | room temperature                                          |
| s        | second or singlet                                         |
| $S_{br}$ | broad singlet                                             |
| SDS      | sodium dodecyl sulfate                                    |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sRNA     | soluble ribonucleic acid                                  |
| SSMD     | strictly standardized mean difference                     |
| SUMO     | small ubiquitin-related modifier                          |
| SVPD     | snake venom phosphodiesterase                             |
| $t$      | time                                                      |
| t        | triplet                                                   |
| TAMRA    | 5-carboxytetramethylrhodamine                             |
| TCEP     | tris(2-carboxyethyl)phosphine                             |
| TEAA     | triethylammonium acetate                                  |
| TEAB     | triethylammonium bicarbonate                              |
| TEMED    | $N,N,N',N'$ -tetramethyl-ethylenediamine                  |
| THPTA    | tris(3-hydroxypropyltriazolyl methyl)-amine               |
| TKI      | thymidine kinase 1                                        |
| TLC      | thin layer chromatography                                 |
| TMP      | trimethylphosphate                                        |
| TOF      | time of flight                                            |
| Tris     | 2-amino-2-(hydroxymethyl)propane-1,3-diol                 |
| tRNA     | transfer ribonucleic acid                                 |
| TrpRS    | tryptophan-tRNA synthetase                                |
| u        | units                                                     |
| Ubc9     | SUMO-conjugated enzyme                                    |
| UV       | ultraviolet                                               |
| v/v      | volume per volume                                         |
| vs.      | versus                                                    |
| w/v      | weight per volume                                         |
| WB       | western blot                                              |
| XRCCI    | X-ray repair cross-complementing protein 1                |
| $\delta$ | chemical shift                                            |
| VI       |                                                           |

# I Theoretical Background

## 1 Interactome of Ap<sub>3</sub>A

### 1.1 Structure and Biological Synthesis of Ap<sub>n</sub>As

Diadenosine polyphosphates (Ap<sub>n</sub>As) are a ubiquitous family of small molecules that have been discovered in various organisms from bacteria to higher eucaryotes.<sup>1,2</sup> They were first detected in the 1960s as part of a study to investigate lysine amino acid activation with lysine-soluble ribonucleic acid (sRNA)<sup>1</sup> synthetase (LysRS) from *E. coli*.<sup>4</sup> Structurally, two adenosines are joined via a 5',5'-phosphoanhydride bridge of varying length. Today, derivatives are known for each size of Ap<sub>n</sub>As ranging from two to seven phosphate groups (see **Figure 1**).<sup>5</sup> The different net charges and conformational states resulting from the diverse phosphate bridges, account for multiple biological functions and a variety of interacting proteins for each Ap<sub>n</sub>A family member.

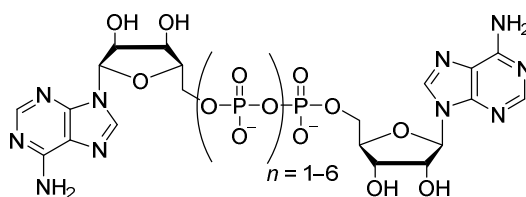


**Figure 1:** Chemical structure of Ap<sub>n</sub>As.

At physiological pH and temperature Ap<sub>n</sub>As prefer the electrostatically stabilized stacked conformation whereas below pH 3 an open conformation was found (see **Figure 2**).<sup>6,7</sup> Ap<sub>n</sub>As are more stable towards hydrolysis than their mononucleotide analogs and undergo only slow hydrolysis between pH 6 and 9.<sup>8,9</sup> Cellular levels of dinucleotides lie in the nano- to micromolar range and are down-regulated by specific and unspecific enzymes.<sup>10</sup> The concentrations, however, were mostly determined over 30 years ago.<sup>11</sup> Intracellular levels seem to play the key role in the diverse biological functions of Ap<sub>n</sub>As, though, detailed mechanisms remain unknown. An early stated correlation between their cytosolic concentration and the state of the cell is now controversially discussed.<sup>9,10,12,13</sup>

Today, the main producers of Ap<sub>n</sub>As *in vitro* and *in vivo* are thought to be aminoacyl tRNA synthetases (aaRS). This was first discovered with a purified lysyl-tRNA synthetase (LysRS) from *E. coli* that produced diadenosine triphosphate (Ap<sub>3</sub>A) and, in larger amounts, adenosine

<sup>1</sup> historical for transfer RNA (tRNA)<sup>3</sup>

tetraphosphate ( $\text{Ap}_4\text{A}$ ), when incubated with adenosine triphosphate (ATP) and lysine in presence of  $\text{Mg}^{2+}$ .<sup>4</sup>

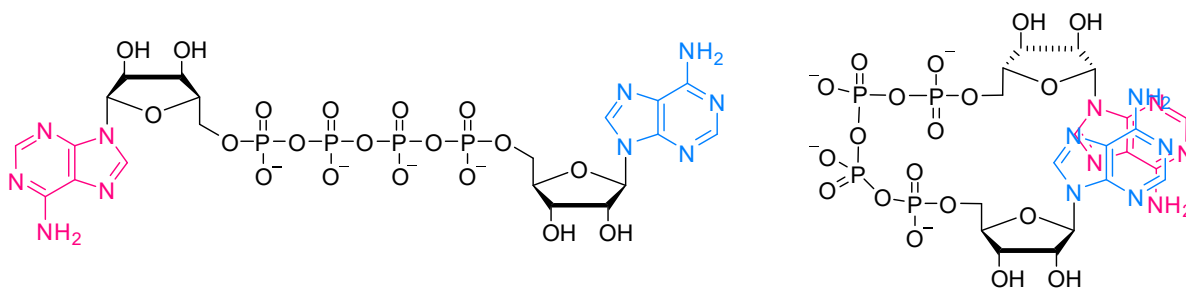


**Figure 2:** Conformations of  $\text{Ap}_4\text{A}$ . Open (left) and stacked (right) conformation of  $\text{Ap}_n\text{As}$  using  $\text{Ap}_4\text{A}$  as example.

The mechanism for the formation of  $\text{Ap}_n\text{As}$  has been discovered as the back reaction of amino acid activation by aaRS and is depicted in **Figure 3**. In a first step, an enzyme-bound aminoacyl adenylate is formed via ATP hydrolysis. Subsequently, the cognate tRNA attacks the activated amino acid. In absence of tRNA, however, ATP can react instead, the adenylate is transferred onto the phosphate bridge and  $\text{Ap}_4\text{A}$  is synthesized. When ATP is exchanged by adenosine diphosphate (ADP) or an adenosine with longer phosphate chains,  $\text{Ap}_3\text{A}$  or higher order  $\text{Ap}_n\text{A}$  family members are formed respectively. Since ATP exists in high intracellular concentrations,  $\text{Ap}_4\text{A}$  is the predominant species among the  $\text{Ap}_n\text{As}$ .<sup>14</sup> During the formation of  $\text{Ap}_n\text{As}$ , the reaction can be reversed, the aminoacyl adenylate can dissociate from the enzyme or be hydrolyzed since the equilibrium is shifted towards pyrophosphorolysis.<sup>2</sup> The presence of cognate tRNAs inhibits  $\text{Ap}_n\text{A}$  synthesis in most cases.<sup>15</sup> There are, however, aaRS like arginyl-tRNA synthetase (ArgRS) and glutamyl-tRNA synthetase (GlnRS) that require the presence of tRNA to synthesize  $\text{Ap}_n\text{As}$ .<sup>15</sup> Furthermore, not every aaRS is able to produce all family members. ArgRS, GlnRS and tryptophan-tRNA synthetase (TrpRS), for instance, are unable to synthesize  $\text{Ap}_4\text{A}$ .<sup>15</sup> Additionally, TrpRS is the only synthetase that can exclusively form  $\text{Ap}_3\text{A}$ .<sup>16,17</sup> In general, overexpression experiments of the various aaRS showed increased levels of the respective  $\text{Ap}_n\text{As}$  in *E. coli* and mammalian cells.<sup>18,19</sup> *In vitro*, further proteins like deoxyribonucleic acid (DNA) ligases,<sup>20,21</sup> firefly luciferase<sup>22</sup>, acyl-coenzyme A (CoA) synthetase<sup>23</sup> and even  $\text{Ap}_4\text{A}$  hydrolase<sup>24</sup> were identified to synthesize various dinucleotides.

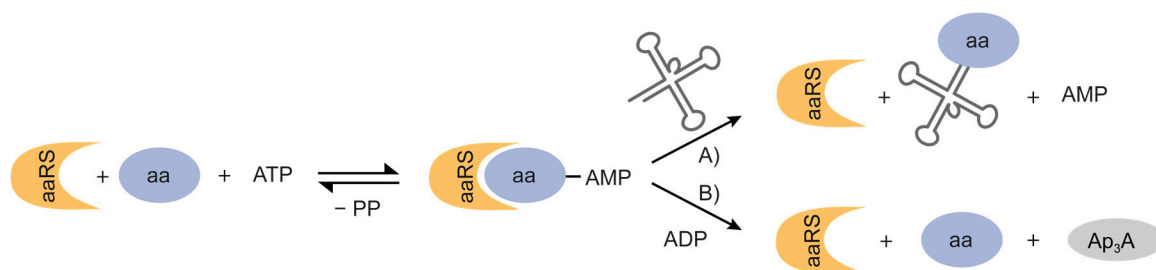


**Figure 3:** Schematic display of the biosynthesis of  $\text{Ap}_n\text{A}$  using  $\text{Ap}_3\text{A}$  as example.

## 1.2 Signaling Pathways of Ap<sub>n</sub>As

Increased levels of Ap<sub>n</sub>As were detected when cells were exposed to external stress stimuli. In prokaryotes heat shock,<sup>25</sup> oxidative stress<sup>26</sup> and exposure to heavy-metal ions<sup>27</sup> lead to a significant accumulation. These elevated levels of Ap<sub>n</sub>As have been associated with a wide range of intra- and extracellular signaling functionalities in which they behave as alarmones or second messengers. In line with this hypothesis, various tissues respond to the extracellular application of Ap<sub>n</sub>As including heart,<sup>28</sup> hippocampus,<sup>29</sup> neutrophils<sup>30</sup> and pancreatic cells.<sup>31</sup> Ap<sub>n</sub>As are also regulators of membrane processes and play a role in both the induction of Ca<sup>2+</sup> oscillations and the control of K<sub>ATP</sub> channels.<sup>15,32</sup> In contrast, Tawfik *et al.* suggested that Ap<sub>4</sub>A might also be a damage metabolite in *E. coli* since they did not observe phenotypic changes upon up- or down-regulation unless extensive amounts of Ap<sub>4</sub>A were produced.<sup>33</sup>

### 1.2.1 Extracellular Signaling of Ap<sub>n</sub>As

Extracellularly, Ap<sub>n</sub>As were found to interact with ligand-gated ion channels (P2X) and G protein-coupled receptors (P2Y) of the purinergic signaling system and even specific diadenosine receptors were identified.<sup>34</sup> Their role as antagonists or agonists at the purinoreceptors is determined by the length of the phosphate chain, whereby ADP is resembled best by Ap<sub>3</sub>A and ATP is mimicked by Ap<sub>4</sub>A.<sup>35</sup> When Ap<sub>n</sub>As in the central nervous system (CNS) are released from vesicles into the synaptic cleft together with other neurotransmitters a fast synaptic transmission can be mediated by targeting P2X receptors (**Figure 4A**).<sup>36,37</sup> In this regard, special functions of Ap<sub>n</sub>As have been revealed in mammalian eyes. Here, they are associated with a regulatory function in the composition of tears,<sup>38</sup> intraocular pressure<sup>39</sup> and wound healing of the cornea.<sup>40</sup> Ap<sub>4</sub>A is even under debate as a potential drug to treat glaucoma.<sup>41</sup> Besides synaptic vesicles, Ap<sub>n</sub>As are also co-stored with ATP in platelet dense granules. Upon activation by thrombin, collagen or ADP, Ap<sub>n</sub>As are released and can lead to either platelet aggregation (Ap<sub>3</sub>A) or contribute to the reversed process of inhibiting aggregation (Ap<sub>4</sub>A) (**Figure 4B**).<sup>42,43</sup> In mammalian hearts, Ap<sub>n</sub>As have been shown to regulate the mitochondrial K<sub>ATP</sub> channel, thereby controlling blood clotting and the vascular tone. Furthermore, administration of Ap<sub>4</sub>A had a positive influence on heart rate and blood pressure and could protect the heart muscle after hypothermic preservation.<sup>44</sup> The beneficial effects of Ap<sub>4</sub>A were reversed when a mitochondrial K<sub>ATP</sub> inhibitor was employed.<sup>45</sup> Different Ap<sub>n</sub>As are potentially responsible for fine-tuning of the myocardial blood flow by fulfilling complementarily either a vasodilator or a vasoconstrictor role.<sup>46</sup>

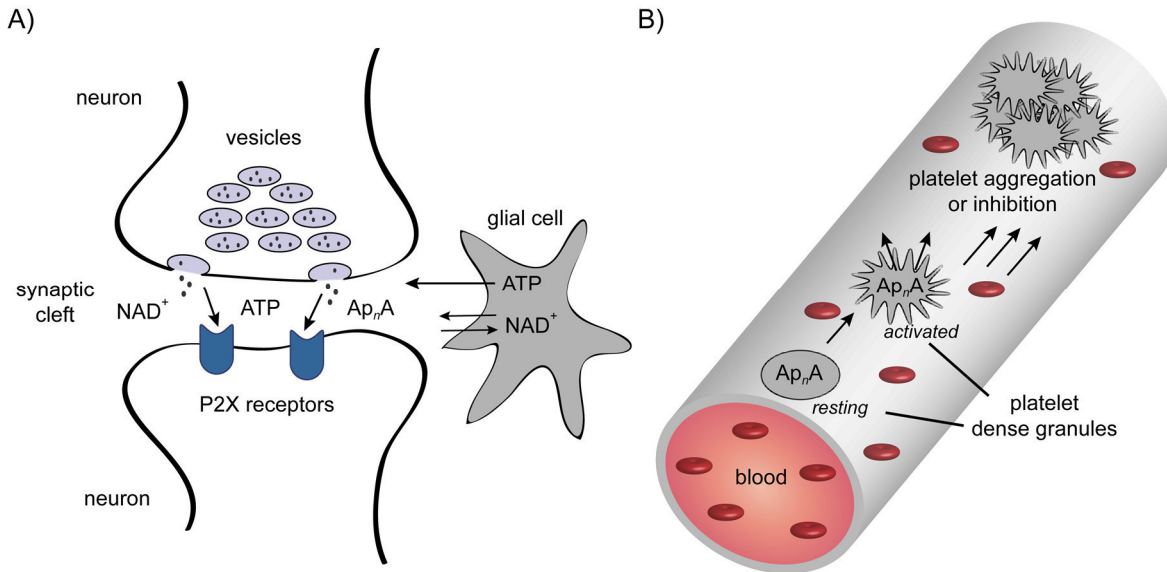


**Figure 4:** Extracellular signaling of Ap<sub>n</sub>As. A) Release of Ap<sub>n</sub>A and other neurotransmitters into the synaptic cleft and mediation of a fast synaptic transmission by targeting P2X receptors. Adenosine nucleotides can further target glial cells which can release purines themselves and target P2X receptors of e.g. neighboring neurons. Figure adapted from<sup>36</sup>. B) Ap<sub>n</sub>As are stored in platelet dense granules and are released upon activation. They contribute, among others, to either platelet aggregation or inhibit this process.

## 1.2.2 Intracellular Signaling of Ap<sub>n</sub>As

### 1.2.2.1 Ap<sub>4</sub>A as Second Messenger

Over the last 30 years, Ap<sub>n</sub>As have been discussed as second messenger molecules. For a molecule to carry this label, Sutherland postulated five criteria as requirements. The definition 'second messenger' is only valid if a molecule fulfills all five of them. (I) The messenger must imitate the effect of an external signal when it is applied intracellularly. (II) The respective enzymes for synthesis and degradation of the messenger must be present. (III) The intracellular concentrations of the messenger must change as a response to the external signal. (IV) Inhibitors of the action of the prospective messenger should block the effect of the external signal and (V) specific targets for the messenger should be present.<sup>47-49</sup>

Ap<sub>4</sub>A has been unambiguously identified as such a second messenger molecule in mast cells. Upon external stimulation with immunoglobulin E-antigen (IgE-Ag), the intracellular concentrations of Ap<sub>4</sub>A increase significantly (III).<sup>50</sup> This is mainly due to phosphorylation at S207 of LysRS upon mitogen-activated protein kinase (MAPK) pathway activation. Phosphorylated LysRS is capable of producing up to six times more Ap<sub>4</sub>A than in an unphosphorylated state and makes it much less efficient at its actual translational activity.<sup>51</sup> It further translocates to the nucleus and forms a tertiary complex with microphthalmia transcription factor (MITF) and histidine triad nucleotide-binding protein 1 (Hint1).<sup>50</sup> Hint1, though, is a specific target for Ap<sub>4</sub>A (V) and is bound and released from the complex by rising amounts of the dinucleotide. Since Hint1 acts as a repressor of transcriptional activity of MITF

in the complex, once it is released, MITF is free to transcribe its responsive genes. Further, it could be shown that degradation of Ap<sub>4</sub>A was conducted by Ap<sub>4</sub>A hydrolase (II).<sup>47</sup> Additionally, the same effect of increased MITF-target expression could be achieved by intracellular application of Ap<sub>4</sub>A via cold-shock or in a more physiological manner by inhibiting Ap<sub>4</sub>A hydrolase (I).<sup>47,50</sup> Since no antagonist of Ap<sub>4</sub>A is available to date, the last of Sutherland's criteria was fulfilled by knocking down LysRS, which indeed decreased Ap<sub>4</sub>A levels in IgE-Ag activated cells significantly and blocked transcription of MITF targets (IV).<sup>52</sup> An overview on Ap<sub>4</sub>A's second messenger activity is displayed in **Figure 5**.

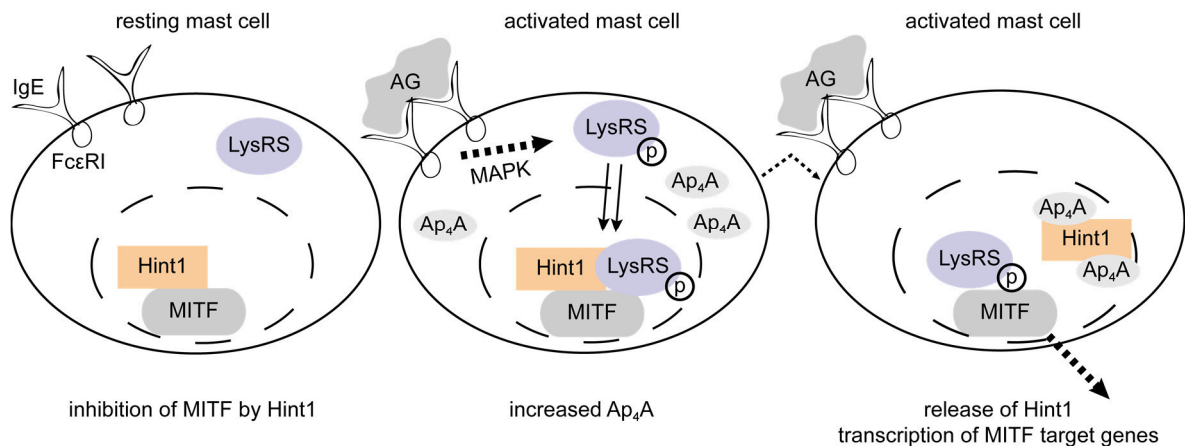


**Figure 5:** Schematic overview on Ap<sub>4</sub>A's second messenger functionality in mast cells. MITF responsive genes are transcribed following IgE-antigen stimulation and increase of Ap<sub>4</sub>A. Figure adapted from<sup>52</sup>.

### 1.2.2.2 DNA Damage Response

Ap<sub>4</sub>A has been studied extensively in context with the DNA damage response. Treatment of cells with non-cytotoxic doses of mitomycin C, a DNA crosslinking agent, lead to a significant increase in Ap<sub>4</sub>A. Furthermore, Ap<sub>4</sub>A was elevated when important DNA repair proteins like X-ray repair cross-complementing protein 1 (XRCC1) or ADP-ribosyltransferase diphtheria toxin-like 1 (ARTD1) were absent, but restored to normal levels upon re-expression of these enzymes.<sup>53</sup> Rising Ap<sub>4</sub>A was therefore associated with stalled replications forks to slow down replication and allow DNA repair as an alternative pathway to common repair mechanisms close to the damage site.<sup>9,53,54</sup> Interestingly, elevated Ap<sub>3</sub>A was not detected in the same experiments. Furthermore, not aaRSs but rather DNA ligases seemed to be responsible for the increased synthesis of Ap<sub>4</sub>A. Ap<sub>4</sub>A, however, was found to be ADP-ribosylated by ARTD1, which might terminate signaling towards inhibition of DNA replication.<sup>53</sup> In case of severe DNA damage, accumulation of further Ap<sub>4</sub>A or different Ap<sub>n</sub>As can result in apoptosis.<sup>13,55,56</sup>

### 1.3 Interaction Partners of Ap<sub>3</sub>A

In general, three different classes of interacting proteins were identified for Ap<sub>n</sub>As, recognizing receptors that are located on the cell surface, enzymes that utilize Ap<sub>n</sub>As as substrates and proteins that bind but do not metabolize the dinucleotides. Several attempts have been made to identify new binding proteins by establishing the respective experiments in cell lysates. The first studies were conducted in the 1990s with Ap<sub>4</sub>A analogs in *E. coli* and delivered several oxidative-stress related proteins and chaperones.<sup>57</sup> Further studies revealed inosine-5'-monophosphate (IMP) dehydrogenase (cell growth regulation) and chaperone SurA<sup>58</sup> next to trehalose-6-phosphate synthase (stress response) and other binders from very diverse protein classes with functions in various biological processes.<sup>59</sup> It still remains unclear, however, if these bacterial proteins are specific Ap<sub>4</sub>A binders or if Ap<sub>4</sub>A only binds as an ATP surrogate.<sup>33</sup> One study was also conducted in murine brain lysate and ATP synthase and several heat shock proteins were identified as Ap<sub>4</sub>A binding proteins.<sup>58</sup> Beyond these experiments aiming directly at the discovery of binding partners, several interacting proteins of Ap<sub>n</sub>As were identified in different studies. Cystathionine beta synthase (CBS) domains of pyrophosphatases, that usually bind different kinds of adenine nucleotides as regulating molecules, bind Ap<sub>n</sub>As in bacteria.<sup>60,61</sup> Thereby, two distinct CBS domains are linked by two AMP-moieties of Ap<sub>n</sub>A. In case of the shorter Ap<sub>3</sub>A, however, only one AMP-moiety is bound tightly.<sup>60,62</sup>

An important landmark in the investigation of Ap<sub>n</sub>As was the discovery of specific degrading proteins, namely the human fragile histidine triad protein (Fhit), an Ap<sub>3</sub>A hydrolase of the histidine triad (Hit) protein family,<sup>63</sup> and the human nucleoside diphosphate-linked moiety X (nudix) motif 2 (NudT2), a protein of the mutator protein MutT family that cleaves Ap<sub>4</sub>A asymmetrically to AMP and ATP.<sup>64</sup> Fhit is also able to cleave other Ap<sub>n</sub>As to a small extent, whereas NudT2 attacks Ap<sub>4</sub>G, p<sub>4</sub>G, Ap<sub>4</sub> and Ap<sub>4</sub>dT next to Ap<sub>4</sub>A.<sup>65</sup> It is widely assumed that these two enzymes are mainly responsible for the balancing of the intracellular levels of Ap<sub>3</sub>A and Ap<sub>4</sub>A as knock-down experiments of the respective genes resulted in significantly increased amounts of Ap<sub>n</sub>As.<sup>9,66,67</sup> Moreover, several binding or processing partners of Ap<sub>4</sub>A were identified, including protein kinases,<sup>68-70</sup> DNA polymerase  $\alpha$ ,<sup>71-73</sup> Aprataxin<sup>74</sup> and Hint 1.<sup>50</sup> Interestingly, both last-mentioned proteins belong to the Hit family, similarly to Fhit. Further interacting proteins of Ap<sub>n</sub>As are unspecific phosphorylases or hydrolases and various receptors, especially from the purinergic signaling system. Overall, several interacting proteins of Ap<sub>n</sub>A were identified over the years, especially for Ap<sub>4</sub>A. In contrast, almost nothing is known about the interaction network of Ap<sub>3</sub>A. Detailed mechanisms of metabolism or transduction signaling, however, remain elusive in all cases.

## 1.4 Affinity-Based Proteomic Profiling (ABPP) of Interacting Proteins

Studies aiming at the isolation and identification of a small molecule's interactome using chemical tools can be divided into two main subgroups. When a specific class of enzymes is under investigation, functional groups can be attached to the probe that only react with active enzymes in the right conformation. These studies are called activity-based proteomic profiling experiments. In contrast, when the whole interactome regardless of binding or processing functionality is studied, affinity-based proteomic profiling (ABPP) is the method of choice. These experiments are solely based on the probe's affinity to interacting proteins. Both approaches require modifications of the respective probes with affinity tags towards solid supports for target enrichment and isolation from the residual protein mixture. Subsequently, target proteins are either analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or directly digested by specific enzymes and finally identified by mass spectrometry (MS).

### 1.4.1 Design of ABPP Experiments

One of the main aspects in ABPP experiments is the design of the small molecule probe. The probe necessarily needs an affinity function for binding to target proteins. Additionally, it has to contain e.g. an affinity tag for immobilization on a solid support (see **Figure 6**). Thereby, the natural binding must ideally not be impaired or only to an acceptable extent.

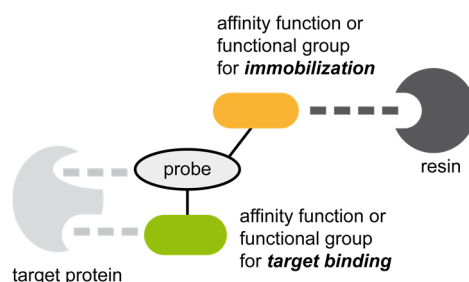


**Figure 6:** Probe design for protein profiling experiments. Different possibilities are available to immobilize the probe on a resin and to bind target proteins.

Several strategies are available for attaching an affinity-based probe (ABP) to a resin (see **Figure 7**). Firstly, functional groups like amines, alcohols, thiols or carboxylic acids can be introduced into the probe for covalent binding to activated matrices, e.g. *N*-hydroxysuccinimide (NHS)- or epoxy-activated sepharose.<sup>75</sup> Furthermore, peptides like the poly histidine-tag and the Strep-tag® can be incorporated and will be recognized by the corresponding Ni or Strep-Tactin, immobilized on a resin.<sup>76,77</sup> Additionally, fluorophore tags with the corresponding antibodies and protein-tags like glutathione-*S*-transferase (GST), binding strongly to glutathione, have been commonly reported.<sup>75,78</sup> The most widely applied strategy utilizes the strong interaction between biotin and a homotetramer of streptavidin.<sup>79</sup> With a  $K_D$  value of  $10^{-14}$  to  $10^{-15}$  M this is

one of the strongest non-covalent interactions known that even resists very stringent conditions like extreme pH values, temperatures and a denaturing environment.<sup>80</sup> Biotin is usually introduced to the probe via a linker to avoid steric interference between the matrix and target proteins and to improve the solubility in aqueous media. Streptavidin-coated beads can also be exchanged by avidin or neutravidin resins, which bind in a similarly strong manner to biotin. The biotin-desthiobiotin is equally bound by streptavidin, however, with a lower  $K_D$  value of  $10^{-11}$  M that allows elution of the probe and binding targets by application of an excess of natural biotin.<sup>81,82</sup> Otherwise, the interaction between biotin and streptavidin can be cleaved by heat denaturation in SDS-containing buffer or by employing 8 M guanidine hydrochloride. In case of a non-covalent interaction between the probe and target proteins, also specific elution with an excess of unmodified probe is possible, thereby releasing the target proteins into solution.

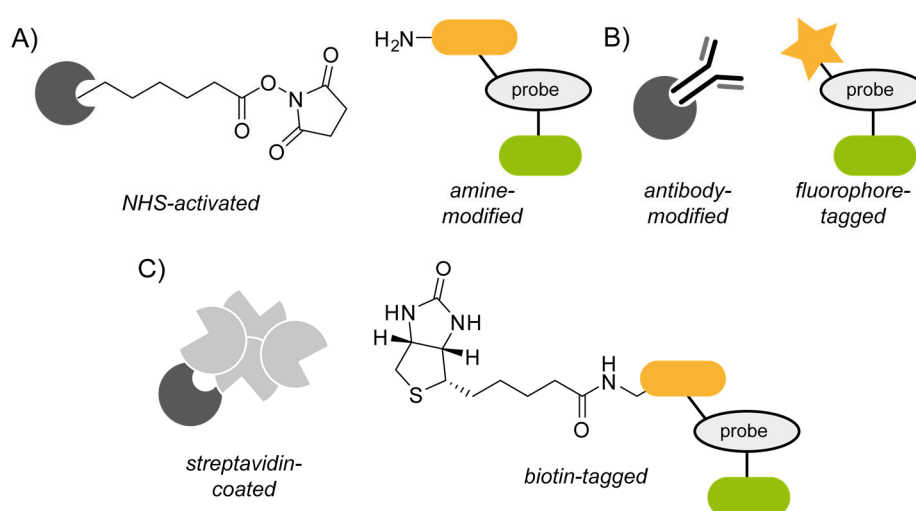


**Figure 7:** Different strategies for immobilization of the probe on a resin. A) NHS-activated resin and amine modified probe. B) Antibody-modified resin and corresponding fluorophore-tagged probe. C) Streptavidin-coated resin and biotinylated probe.

The ABPP experiment can either be started by immobilizing the probe on the resin, followed by incubation with protein mixtures, or vice versa. However, the attachment of biotin to a small molecule might reduce cell permeability significantly and the additional introduction of a polar linker might be considered when ABPP inside living cells is requested. In most cases, cell lysates are sufficient for a first indication of interaction partners. If the conditions of cell lysis, however, disturb the binding of targets and no proteins are isolated, intact cells should be taken into consideration as well.<sup>83</sup> To further improve binding between probe and targets, studies can be performed with the help of crosslinkers, e.g. functional groups like sulfonyl chlorides or active esters, specifically targeted by lysines. In affinity-based experiments the application of a photoaffinity tag that reacts mainly unspecifically to residues in close proximity is an often utilized approach.<sup>84</sup> Covalent bonds allow more stringent washing conditions after immobilization to remove non-specifically binding proteins to either the probe, the affinity tag or the resin. Additionally, target proteins stay attached to the probe under denaturing conditions of SDS-PAGE and transient interactions can be stabilized. When a trifunctional

probe is utilized an additional fluorophore allows the easy visualization of proteins by fluorescence read-out. Fluorescent labels can also be attached after incubation with protein extracts by copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC, see 1.4.1.2) when an alkyne handle had been incorporated in the probe. This small bioorthogonal modification might have a lesser impact on the probe and also allows the incorporation of an affinity tag at a later stage of the ABPP procedure.

The removal or identification of unspecifically binding proteins is a crucial step during ABPP experiments. Therefore, depending on the affinity between probe and target proteins, the ideal washing strategy after immobilization needs to be developed in order not to lose low-affinity targets. Specific elution with an unmodified version of the probe is the best way to separate potential targets from residual proteins on the resin. Limitations of this method are the need for solubility of high amounts of unmodified probe, slow dissociation kinetics of probe<sup>85</sup> and protein and the non-covalent binding to target proteins. Boiling in SDS buffer on the other hand releases all bound proteins, regardless of their specificity to the probe.

Hence, a series of control experiments is indispensable (see **Figure 8**). The most important control is the “bead only” reaction, in which no probe is applied and only proteins are identified that bind unspecifically to the resin.<sup>86</sup> In this way, false positives will be discovered and characterized as background in every sample. However, there might be target proteins that also stick to the beads, so there is a chance that specifically binding proteins are missed on grounds

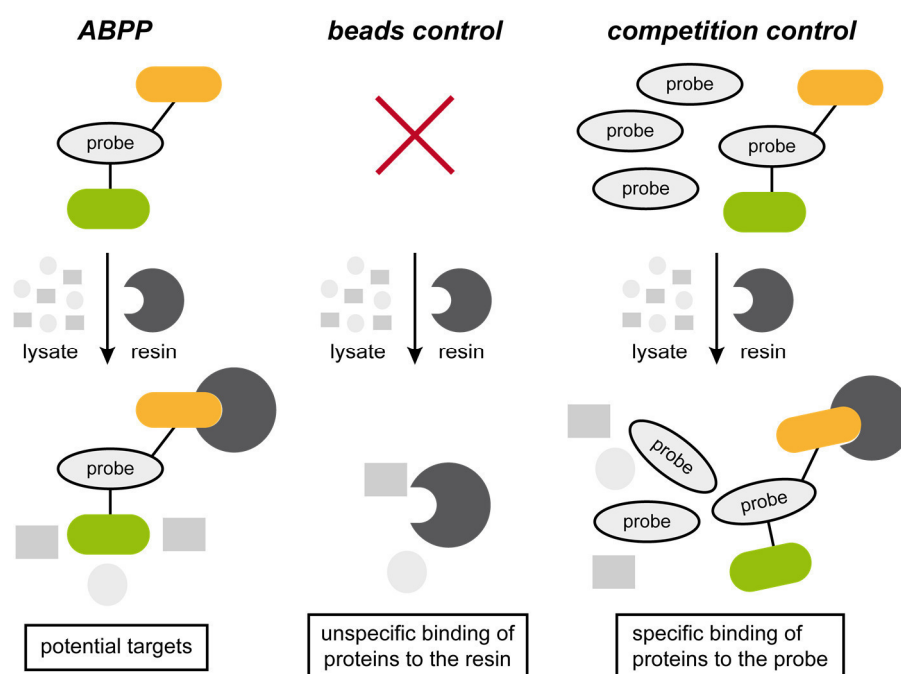


**Figure 8:** Overview on important control experiments. The beads control in which no probe is applied and unspecific binding of proteins to the solid phase is studied. A competition control in which an unmodified probe is employed to find specifically binding proteins by comparing the ABPP and competition control. Proteins missing in the competition control are considered potential targets.

of this control. Another important control is a competition experiment. Thereby, an excess of an unmodified probe is incubated with cell lysate or intact cells before the probe is added and ABPP is performed.<sup>87-89</sup> All proteins absent in the competition control but present in the actual ABPP sample are considered as targets. These results can also be compared to isolated proteins by SDS-PAGE analysis where the indicative targets should be absent or at least apparent in lower intensity in the competition control. By simply splitting proteins into groups of presence and absence, however, targets again may get lost. Quantitative proteomics with detection of the relative abundance of isolated proteins, together with sufficient replications of the experiments will enhance the chances of identifying true targets. Further controls include samples with modified probes, where the functionalities necessary for ABPP are attached in positions that significantly lower the probe's activity itself or in positions that are not tolerated by already known processing enzymes.<sup>90,91</sup> An additional aspect worth considering is the concentration of the probe. In general, the probes will primarily find their physiological targets and bind with high affinity, but might also interact with other proteins when applied in high concentrations. Otherwise, specific targets might be of low abundance and difficult to detect when probe concentrations are too low. Furthermore, it is almost impossible to estimate the relative affinity towards unknown targets and therefore very difficult to assess the optimal probe concentration. At worst, too much probe can lead to the mere detection of highly abundant background proteins. To further identify background proteins independent from control experiments, so called "black-lists" are available that collect commonly occurring background proteins from different ABPP experiments with various probes.<sup>92</sup> A similar list exists for proteins reccuringly appearing in bead controls with different resins.<sup>93</sup> Another option to minimize non-specifically binding proteins is to expose the lysate to a control matrix prior to the actual experiment. Thereby, background proteins will stick to the resin and the protein extract applied in ABPP will be cleared of these unspecific binders.<sup>94</sup>

Overall, probe design and concentration, lysate concentration, washing, elution and competition conditions need to be optimized to obtain the lowest amount of background proteins possible and to design a successful ABPP experiment.

### 1.4.1.1 Photoaffinity Labeling

Photoaffinity labeling offers the possibility to covalently capture interacting proteins in ABPP experiments. The irradiation with ultraviolet (UV) light forms reactive intermediates from photoaffinity groups that non-specifically react with double bonds or insert into C-H and N-H sites. The mainly applied functionalities are aryl azides, diazirines and benzophenones.<sup>95</sup> Upon photoactivation with long-wave UV light, aryl azides release N<sub>2</sub> and form nitrenes (**Figure 9A**) that react with all kinds of bonds in close proximity. In contrast to alkyl or acyl azides, aryl azides are more stable due to mesomeric stabilization and have a higher photoefficiency. Next

to the insertion into C-H or heteroatom-H bonds, a ring expansion followed by nucleophilic attacks of primary amines is also a possibility. Thus, buffers containing free amines should be omitted in order to prevent quenching of the reaction. Additionally, the presence of thiol-containing reducing compounds must be avoided to prevent reduction of the azide to an amine, thereby impeding the photoreaction.<sup>96</sup> Activation of diazirines yields reactive carbenes after loss of N<sub>2</sub> upon irradiation with long-wave UV light (**Figure 9B**). They are more stable than aryl azides, their activation is more efficient and due to their small size, they can be versatilely incorporated without steric hindrance of their surroundings. On grounds of this, to date they are more and more commonly utilized.<sup>97</sup> Activated benzophenones contain a diradical to react with neighboring protein backbones or functional groups of the amino acid side chains. They can repeatedly be activated during irradiation after returning to their ground state in case of no reaction. Hence, benzophenones have a higher crosslinking efficiency compared to aryl azides and diazirines.<sup>98</sup>

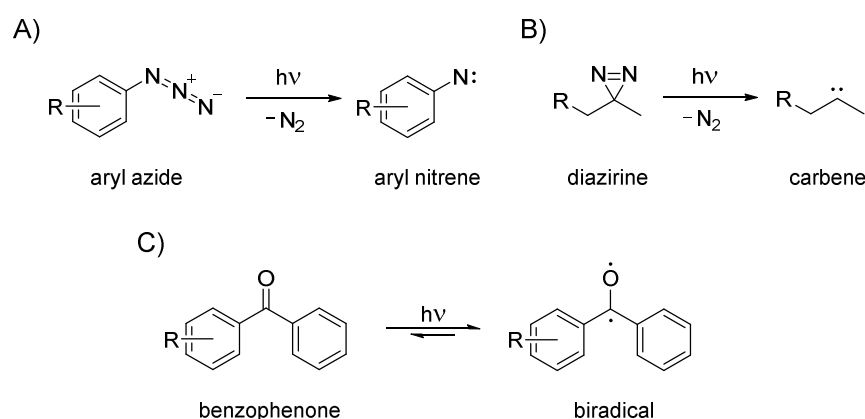


**Figure 9:** Structures of three commonly applied photocrosslinkers in ABPP experiments and their activated states.

Several photoaffinity labeling reactions have been employed with Ap<sub>n</sub>As or mono adenosine polyphosphates. The introduction of an azide at position C8 of the adenosine moiety offers an easy access to the photoactivatable aryl azide functionality and has been successfully incorporated into Ap<sub>3</sub>A,<sup>99</sup> Ap<sub>4</sub>A<sup>57,73,99,100</sup> and ATP.<sup>101,102</sup> Benzophenones and diazirines have already been applied in ATP, however, not yet installed in diadenosine polyphosphates.<sup>103,104</sup>

#### 1.4.1.2 Copper(I)-Catalyzed-Azide-Alkyne-Cycloaddition (CuAAC)

Based on the development of the 1,3-dipolar cycloaddition between azides and alkynes reported by Huisgen in the 1960s (**Scheme 1A**),<sup>105</sup> the groups of Sharpless<sup>106</sup> and Meldal<sup>107</sup> presented a considerably accelerated reaction by adding catalytic amounts of copper(I) (Cu<sup>I</sup>) 40 years later. The resulting 1,4-triazole is obtained regioselectively, whereas the reaction under catalysis of a ruthenium complex solely delivers the 1,5-triazole.<sup>108,109</sup> The uncatalyzed reaction, however, provides a mixture of both isomers. CuAAC proceeds under aqueous and atmospheric



depicted in **Figure 10**: *in-gel* (A), *in-solution* (B) and *on-beads* (C) digestion.<sup>112</sup> The first method needs to be pursued when the proteins are eluted from the beads by heating in SDS-loading dye since trypsin digestion is not compatible with reducing agents. After separation by SDS-PAGE respective bands are subsequently excised, the proteins digested *in-gel* and the resulting peptides from the supernatant are subjected to MS analysis. In case of specific elution of proteins or by treatment with excess of biotin when desthiobiotin is attached to the probe, the digestion can be performed *in-solution*. This results in a broader mixture of peptides compared to *in-gel* digestion since all potential target proteins are digested at once and not only a small fraction from the gel. Another option is the *on-beads* digestion for which the proteins are

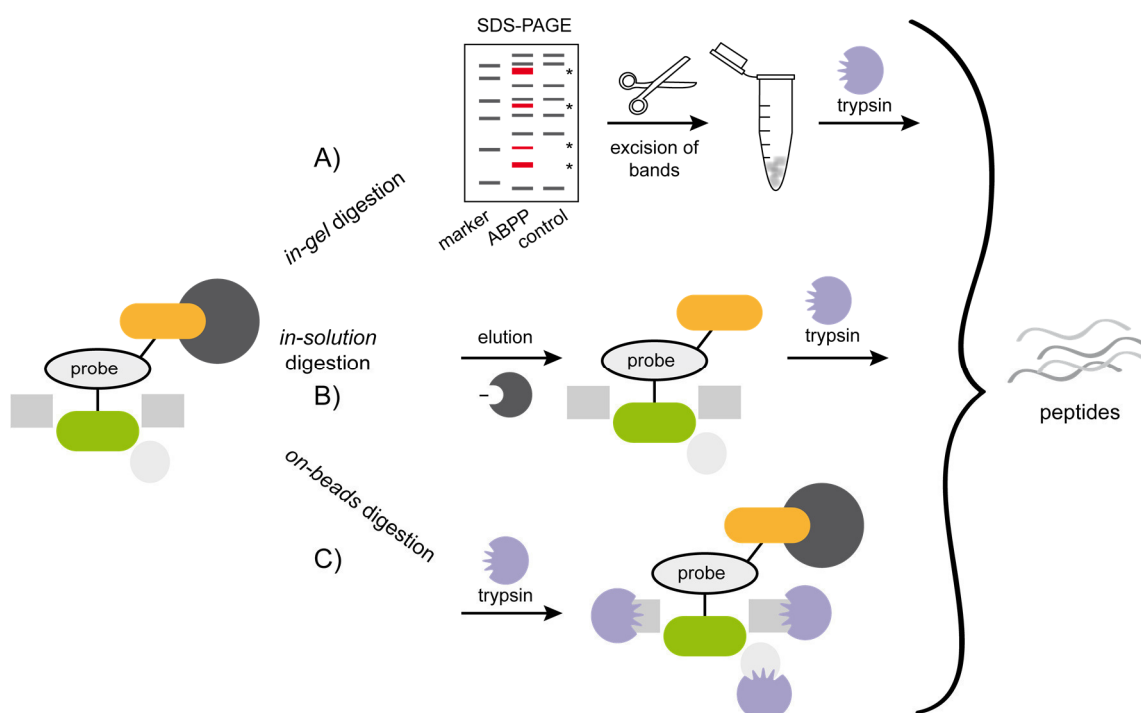


**Figure 10:** Various possibilities of digestion of targeted proteins after affinity enrichment. A) *In-gel* digestion. B) *In-solution* digestion. C) *On-beads* digestion.

digested directly on the resin, without prior elution. This also results in a complex peptide mixture but the extra step of SDS-PAGE is omitted when specific elution is not possible due to covalent binding between probe and target. Subsequently the peptides are separated by high performance liquid chromatography (HPLC) and injected to the mass spectrometer (**Figure II**).<sup>113</sup> In a first step, peptide ions are formed and further separated by mass to charge ratio ( $m/z$ ). These can then be further fragmented, e.g. by collision-induced dissociation (CDI)<sup>114</sup> or higher-energy collisional dissociation (HCD),<sup>115</sup> separated again and detected in a second step in an ion trap mass analyzer (e.g. orbitrap).<sup>116</sup> Fragmentation yields a specific pattern of each peptide that can be used to identify the protein it originates from via data base search.

Relative quantification of proteins from different samples can be performed with MS by either using stable isotopes for metabolic<sup>117,118</sup> or chemical labeling<sup>119</sup> or label-free quantification.<sup>120,121</sup> In the label-free analysis the MS signal of each peptide is integrated and compared to the

integral of the same peptide in another MS run. The values for peptides assigned to the same protein are combined and relative quantification can be performed. This method is less expensive and elaborate and not limited to an increasing complexity of the sample in comparison to the application of stable isotopes.<sup>122</sup>

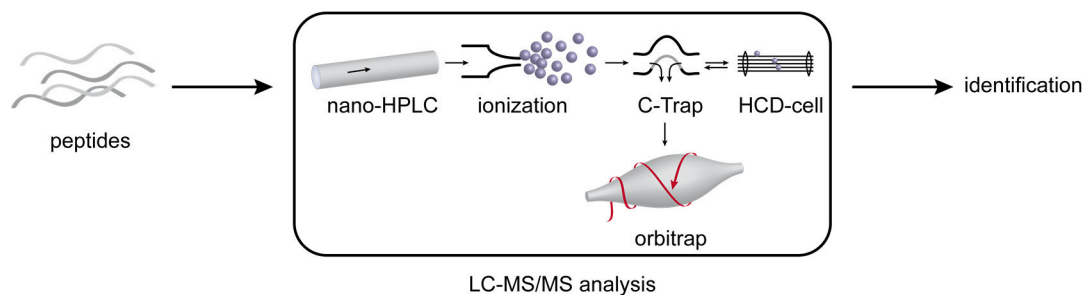


**Figure 11:** Analysis of peptides by LC-MS/MS. Separation of peptides by nano-HPLC, subsequent ionization, MS analysis and identification of the respective proteins.

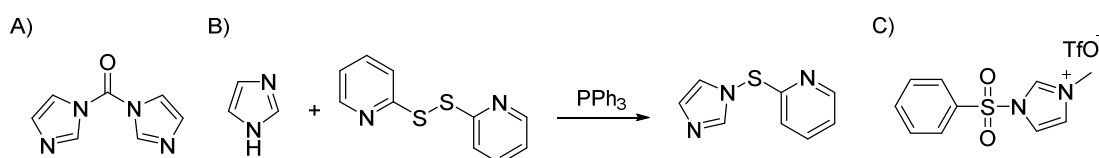
## 1.5 Chemical Syntheses of Ap<sub>3</sub>A

The syntheses of dinucleoside polyphosphates (Np<sub>n</sub>Ns) suffer from several disadvantages in comparison to the generation of open-chain nucleotides. To start with, they contain the double amount of reactive functional groups that can undergo undesired side reactions. Therefore, time-consuming protecting strategies during the synthesis might become necessary. Due to their highly charged phosphate chains dinucleotides need to be purified elaboratively via ion-exchange and reversed-phase chromatography. In some cases, reactive intermediates cannot be isolated and purified at all, but need to be further employed in the next reaction right away. Unstable intermediates also account for the need of anhydrous reaction conditions.<sup>123</sup> The solubility of the nucleotides in organic solvents, in turn, can be challenging, but the exchange of counterions to tri- or tetraalkyl ammonium salts solves this problem in most cases.<sup>123,124</sup> Despite all of these drawbacks, successful syntheses of dinucleotides and especially Ap<sub>n</sub>As were reported, albeit in relatively low yields. Syntheses were performed starting from P<sup>III</sup> and P<sup>V</sup> species, whereby the higher reactive P<sup>III</sup> species need to be converted into the natural occurring P<sup>V</sup> forms. Even enzymes have been utilized to produce Np<sub>n</sub>Ns, although these reactions are limited by scale and substrate specificity.<sup>125-127</sup>

### 1.5.1 Syntheses of Hydrolyzable Ap<sub>3</sub>A

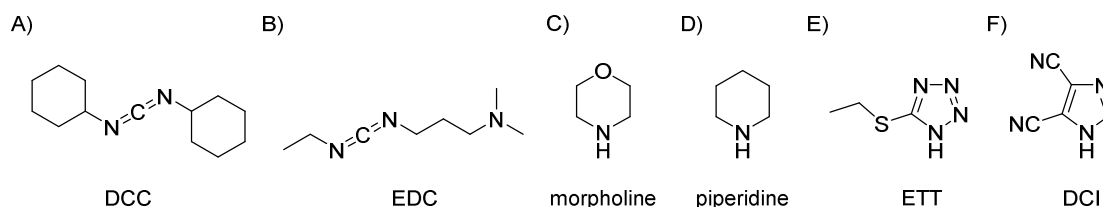
The most common chemical preparation of hydrolyzable Ap<sub>3</sub>As starts from P<sup>V</sup> species and involves the condensation of two nucleotides. Since phosphates themselves are too unreactive, they need to be activated to be attacked by nucleophiles in a donor-acceptor manner. 5'-Phosphoromorpholidates,<sup>35,128</sup> 5'-phosphoroimidazolides,<sup>129</sup> 5'-phosphoropiperidates<sup>130</sup> and

further activated phosphates have been utilized as donors. In general, phosphorimidazolides are most frequently used due to their stability compared to other activated phosphates.<sup>129</sup> Typically, phosphates are reacted with *N,N'*-carbonyldiimidazole (CDI, **Scheme 2A**) to generate the active imidazolide species.<sup>35,131</sup> A further convenient method to generate phosphorimidazolides utilizes triphenylphosphine, 2,2'-dithiodipyridine and imidazole (**Scheme 2B**).<sup>132</sup> Recently, 1-methyl-3-benzenesulfonylimidazolium triflate was developed as a sulfonyl imidazolium reagent (**Scheme 2C**).<sup>133</sup>



**Scheme 2:** Different imidazolide activation reagents. A) Structure of carbonyldiimidazole (CDI). B) Scheme for the preparation of an imidazolide activation reagent from imidazole, dithiodipyridine and triphenylphosphine (PPh<sub>3</sub>). C) Structure of 1-methyl-3-benzenesulfonylimidazolium triflate as activation reagent.

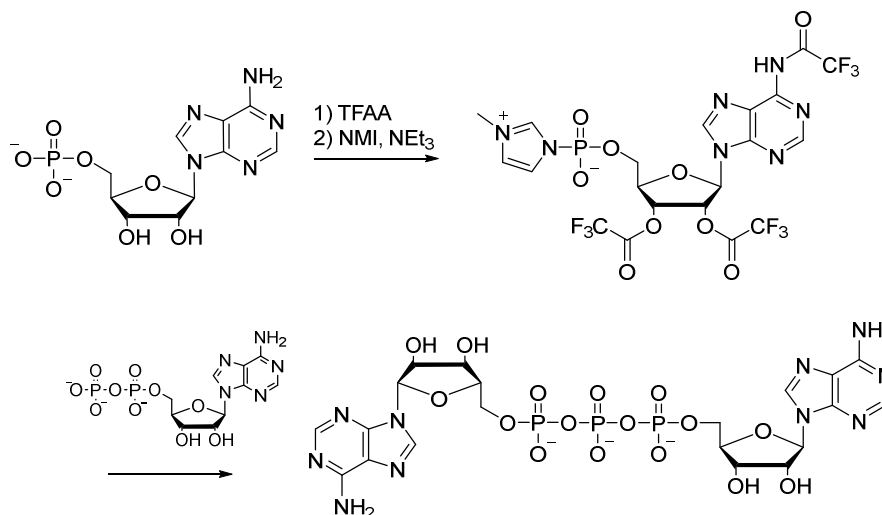
Other activation strategies involve *N,N'*-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC),<sup>134</sup> morpholine,<sup>35,128</sup> piperidine or derivatives of these (see **Scheme 3A–D**). Additives in the coupling step could improve reaction yields and times. Common catalysts include tetrazole, 5-(ethylthio)-1*H*-tetrazole (ETT) or 4,5-dicyanoimidazole (DCI) (**Scheme 3E, F**), which vary concerning their acidity and nucleophilicity.<sup>135–137</sup> Lewis acids like ZnCl<sub>2</sub> can activate the P–N bond, thereby contributing to a higher turnover.<sup>138</sup>



**Scheme 3:** Structures of different activating reagents (A–D) and catalysts (E, F).

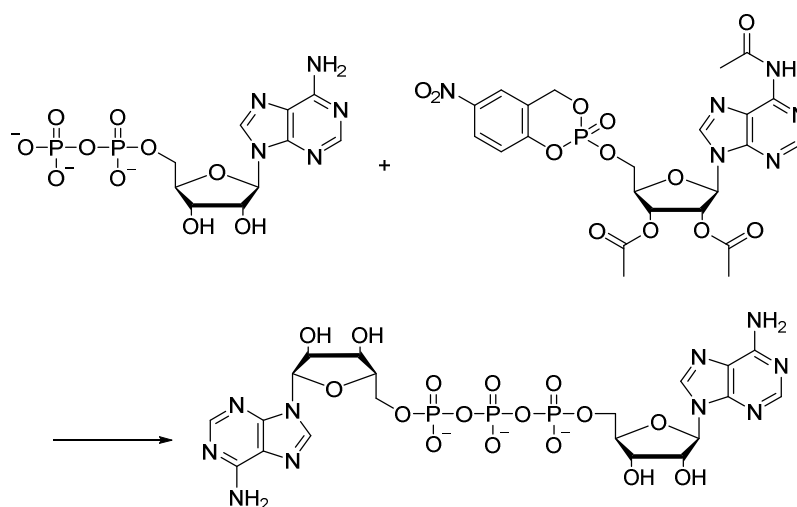
A few different methods have been employed to synthesize hydrolyzable Ap<sub>3</sub>A from activated P<sup>V</sup> species. One common procedure was developed by Mohamady and Taylor and includes the *in situ* protection of the NMP with trifluoroacetic anhydride (TFAA). Basic activation with *N*-methylimidazole (NMI) and coupling in DMF is followed by basic cleavage of the TFA protecting groups in protic solution to give Ap<sub>3</sub>A (**Scheme 4**).<sup>139</sup> In contrast to the original approach developed for deoxyribonucleotide triphosphates (dNTPs),<sup>140</sup> sodium salts were applied instead of tributyl ammonium salts and a considerably higher excess of TFAA was used to absorb excess water. In this way, time-consuming pre-drying of the starting materials could be avoided.<sup>141</sup> Sun *et al.* recently developed a procedure utilizing nucleoside phosphoropiperidates, DCI and *N*-methylpyrrolidone as solvent.<sup>130</sup> Another strategy involves 1-methyl-3-benzenesulfonylimidazolium triflate and NMI for activation and ZnCl<sub>2</sub> as catalyst in the coupling step.<sup>133,142</sup> ADP-imidazolide was utilized by Sawai *et al.* in reaction with AMP under catalysis of Mn<sup>2+</sup> in *N*-ethylmorpholine buffer.<sup>143</sup>

Disadvantages of  $P^V$  utilizing syntheses are the long reaction times, the high excess of reagents and the need to find the specific pair of activator and catalyst for each reaction. Therefore,  $Ap_3A$  syntheses were developed based on  $P^{III}$  chemistry. Initially designed for solid-phase synthesis,<sup>144</sup>  $P^{III}$  species readily react with nucleophiles under slightly acidic conditions.<sup>145,146</sup> However, an additional oxidation step is necessary to obtain the final  $P^V$  compound.

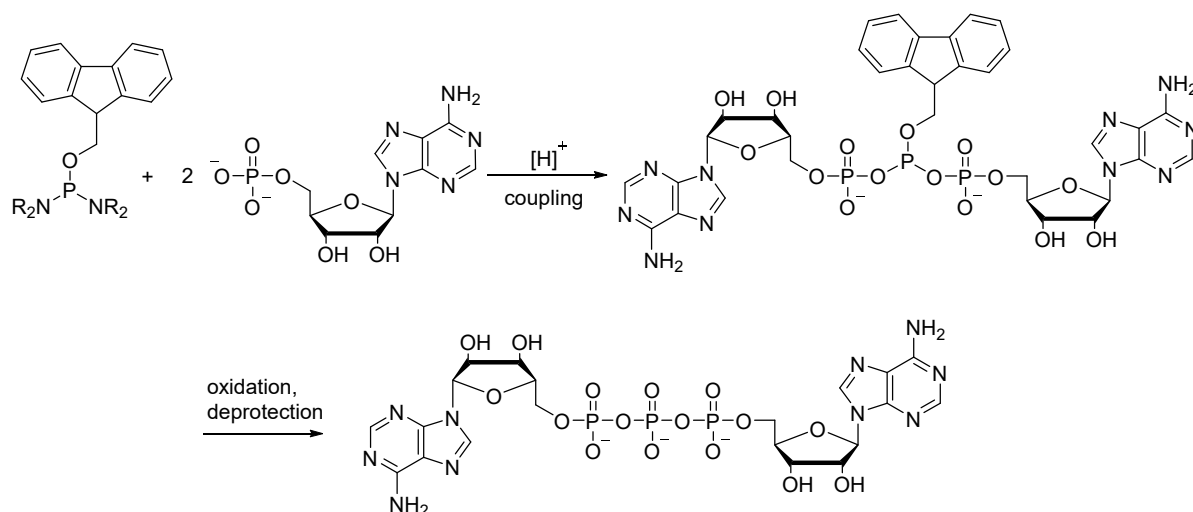


**Scheme 4:**  $Ap_3A$  synthesis from *N*-methylimidazole activated AMP and ADP.

The *cycloSal* phosphotriester intermediate was developed by the Meier group as active ester and pronucleotide for the synthesis of a wide range of nucleotides. The attack by ADP on an adenosine precursor yields  $Ap_3A$  (**Scheme 5**).<sup>147-149</sup> Jessen *et al.* designed a novel method based on phosphordiamidites. It allows fast access to  $Ap_3A$  without nucleobase protection or nucleotide activation under ambient conditions. The bis(9-fluorenylmethyl)-protected phosphordiamidite is treated with two equivalents of nucleotide under weak acidic conditions, oxidized by peroxides, deprotected with piperidine and precipitated without further purification (**Scheme 6**).<sup>145,146,150</sup>



**Scheme 5:** *CycloSal* approach developed by Meier *et al.* to obtain  $Ap_3A$  from ADP and *cycloSal*-modified AMP.



**Scheme 6:** Phosphordiamidite approach developed by Jessen *et al.* to synthesize hydrolyzable Ap<sub>3</sub>As without nucleobase protection.

### 1.5.2 Syntheses of Non-Hydrolyzable Ap<sub>3</sub>A

The nucleophilicity of water is already sufficient to cleave the phosphate bridge of Ap<sub>3</sub>A between P<sub>α</sub> and P<sub>β</sub>. Although the hydrolysis between pH 6 and 9 is slow ( $k_{\text{obs}} = 1.5 \times 10^{-7} \text{ s}^{-1}$ ), in presence of specific and non-specific hydrolases and phosphorylases Ap<sub>3</sub>A is readily cleaved.<sup>8</sup> The investigation of Ap<sub>3</sub>A in a biological context therefore requires analogs that are stable under various conditions. Thus, the labile phosphate chain must be modified in order to withstand hydrolysis. The synthetic strategies include the introduction of a methylene or halomethylene bridge<sup>151,152</sup> instead of a bridging oxygen and the substitution of a non-bridging oxygen by higher chalcogens<sup>153,154</sup> or a borane group.<sup>155</sup> All of these modifications have impact on the natural characteristics of Ap<sub>3</sub>A. The difluoromethylene bridge, e.g., is supposedly the best mimicry of the natural oxygen bridge in terms of stereoelectronics, however, the fluorine atoms might disturb binding to an interacting protein in a different way, e.g. via different hydrogen bonds. Therefore, different derivatives might be ideally suited to study varying biochemical tasks. An overview on reported Ap<sub>3</sub>A analogues that withstand hydrolysis or have reduced hydrolytic rates in comparison to the natural compound is depicted in **Figure 12**.

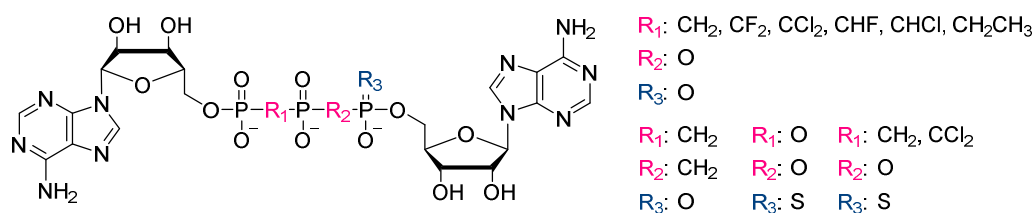
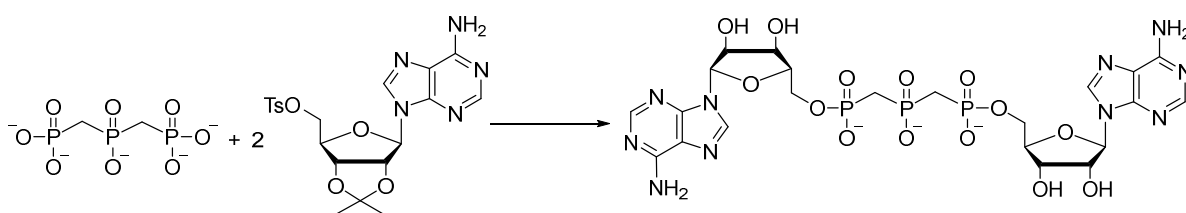


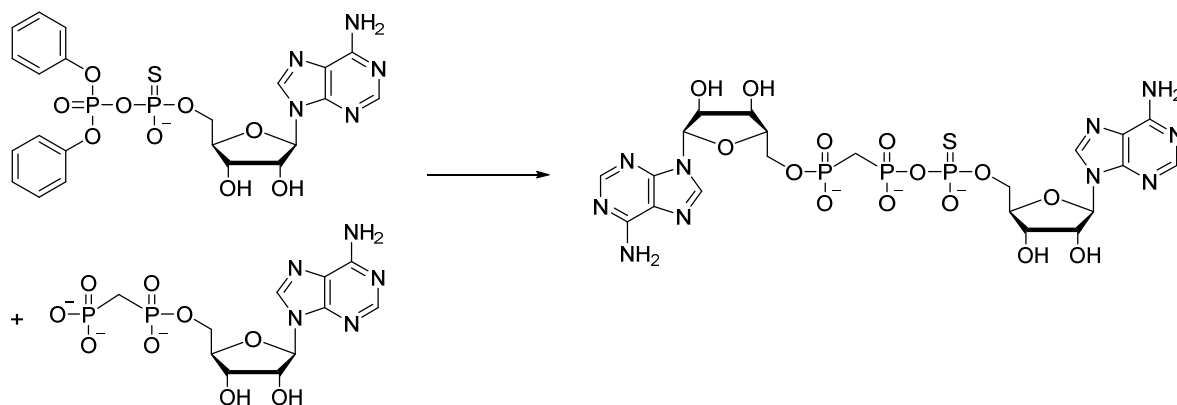
**Figure 12:** Overview on synthesized non-hydrolyzable Ap<sub>3</sub>A derivatives.

Synthetically, non-hydrolyzable analogs of Ap<sub>3</sub>A bear different challenges than their hydrolyzable counterparts. In principal, the same strategies are applicable, however, imidazole

activation e.g. is not compatible with thiophosphates.<sup>156</sup> Blackburn *et al.* synthesized the first bismethylene analog by reaction of bis(dihydroxyphosphinomethyl)phosphinic acid with *O*-isopropylidene adenosine 5'-tosylate (**Scheme 7**).<sup>157</sup> Later, Klein *et al.* improved the synthesis by condensation of 2',3'-*O*-benzylidene-6-azidoadenosine and bis(*O*-benzylphosphonomethyl)phosphinic acid benzyl ester with  $\text{PPh}_3$  and diethyl azidocarboxylate and subsequent hydrogenolysis.<sup>158</sup> The first mixed non-hydrolyzable  $\text{Ap}_3\text{A}$  analog was also synthesized by Blackburn *et al.* by condensation of a 5'-thiomonophosphate with a 5'-methylene bisphosphonate (**Scheme 8**).<sup>159</sup> Diphenyl phosphorochloridate<sup>160</sup> was chosen as activating reagent for the thiophosphate which was then reacted with an excess of the respective diphosphate. The obtained diastereomeric mixture could not be separated but proved to be non-hydrolyzable by the specific  $\text{Ap}_3\text{A}$  hydrolase Fhit.



**Scheme 7:** Synthesis of bismethylene-bridged non-hydrolyzable  $\text{Ap}_3\text{A}$ .



**Scheme 8:** Synthesis of mixed non-hydrolyzable  $\text{Ap}_3\text{A}$ .

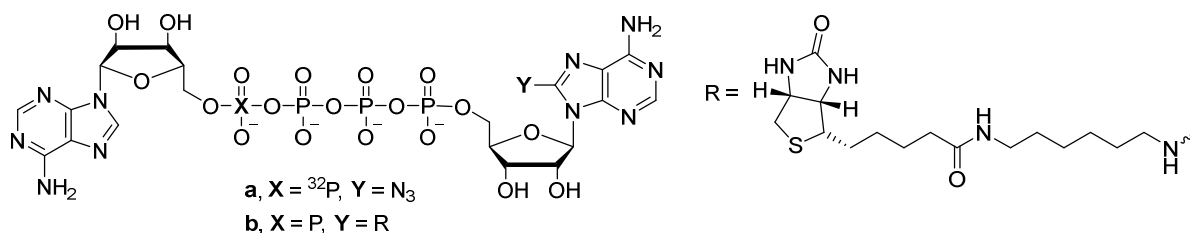
Another set of  $\text{Ap}_3\text{A}$  analogs was synthesized that contained a thiophosphate at  $\text{P}_\alpha$  and  $\text{P}_\beta$  or only one methylene or halomethylene group.<sup>157</sup> The Jessen group further claims that their phosphordiamidite procedure is suitable for thiophosphate analogs as well by applying  $\text{S}_8$  instead of other oxidizing agents in the final oxidation step.<sup>146</sup>

### 1.5.3 Syntheses of Modified $\text{Ap}_n\text{As}$ to Identify Interacting Proteins

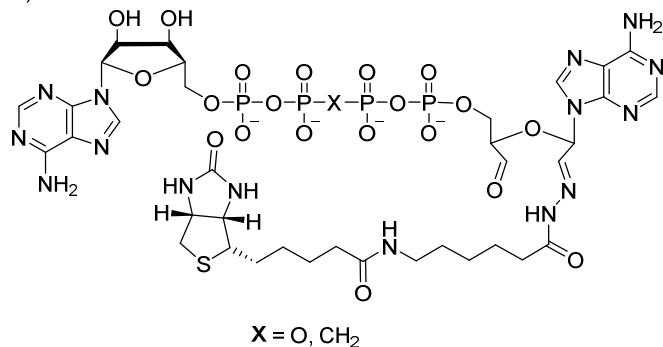
Several hydrolyzable  $\text{Ap}_4\text{A}$  derivatives have been utilized in cellular experiments to identify interacting proteins in bacteria or mammalian cells.  $^{32}\text{P}_\alpha$ -labeled 8- $\text{N}_3$ - $\text{Ap}_4\text{A}$  (**Scheme 9Aa**) was synthesized for photocrosslinking studies in *E. coli* lysates from  $^{32}\text{P}$ -ATP and 8- $\text{N}_3$ -AMP with

activation via EDC.<sup>57</sup> In another study, ATP was treated with NaIO<sub>4</sub>, reacted with 6-hydrazidohexyl D-biotinamide, reduced and enzymatically coupled to ATP with LysU to give a biotinylated Ap<sub>4</sub>A analog at the ribosyl side (**Scheme 9B**), which was applied in experiments in *E. coli* and murine brain lysate.<sup>58</sup> A similar compound modified with a methylene bridge between P<sub>β</sub> and P<sub>γ</sub> (**Scheme 9B**) was tested in *E. coli*<sup>59</sup> as well as 8-(6-aminohexyl)-amino-Ap<sub>4</sub>A-biotin (**Scheme 9Ab**), which was synthesized from the respective N8-biotinylated ATP and adenosine 5'-phosphosulfate using Apa2,<sup>24</sup> a yeast Ap<sub>4</sub>A phosphorylase.<sup>33</sup>

A)



B)



**Scheme 9:** Structures of Ap<sub>4</sub>A probes utilized in various ABPP experiments.

## 2 Small Molecule Inhibitors of the Tumor Suppressor Fhit

### 2.1 Fragile Histidine Triad Protein (Fhit)

The human Fhit protein was discovered in 1996 by Ohta *et al.* when the gene, affected by the translocational breakpoint t(3;8) (p14.2;q24), was identified.<sup>62</sup> In 1979 this chromosomal translocation was found responsible for a recurring familial renal cell carcinoma in an Italian-American family.<sup>63,161,162</sup> The respective gene, located on 3p14.2, was identified as *FHIT*, expressing a protein with 69% similarity to the Ap<sub>4</sub>A-asymmetrical hydrolase from *Schizosaccharomyces pombe*.<sup>62</sup> Fhit is a branch 2 member of the human Hit protein family that consists of seven proteins sharing a conserved His-X-His-X-His-XX (Hit) motif, with a hydrophobic amino acid X.<sup>163</sup> As a small protein, it consists of 147 amino acids that forms a homodimer *in vivo*. In this form, the protein binds two substrate molecules of Ap<sub>3</sub>A and hydrolyzes them to AMP and ADP.<sup>164</sup> From the crystal structure of Fhit in complex with a non-hydrolyzable Ap<sub>3</sub>A analog (see **Figure 13**), an extensively negatively charged surface is revealed due to bound Ap<sub>3</sub>A at the protein surface.<sup>165</sup>

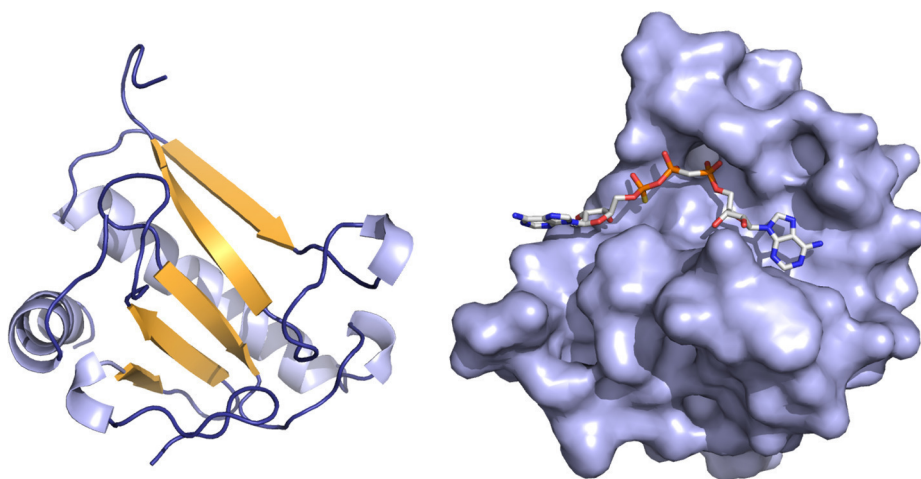


**Figure 13:** Crystal structures of Fhit (left, PDB: 4FIT<sup>166</sup>) and in complex with a non-hydrolyzable Ap<sub>3</sub>A analog (right, PDB: 1FHI<sup>165</sup>).

### 2.2 Genome Caretaker

*FHIT* contains one of the most active common fragile sites (CFSs) of the human genome, FRA3B. These conserved chromosomal regions are sensitive to replication stress and are reported to be involved in the development and progression of many types of cancer.<sup>167</sup> Typical mutations on CFSs include deletions, translocations and promoter methylation resulting in reduced or lost expression of the respective encoded proteins. *FHIT* was described as the second most regularly deleted gene among human cancers.<sup>168,169</sup> During cancer progression,

genomic instability is detected throughout the whole genome and is therefore an elaborative indicator of cancer.<sup>170,171</sup> A genomic caretaker is defined as a gene that is highly involved in the DNA damage repair and hence, combats genomic instability.<sup>172</sup> Fhit might be such a genomic caretaker since its loss of expression is one of the earliest events observed in cancerous tumors.<sup>173</sup> Non-regular expression of the Fhit protein has been observed in almost all common carcinomas including head and neck,<sup>174</sup> gastrointestinal,<sup>175</sup> cervical,<sup>176</sup> lung,<sup>177</sup> breast<sup>178</sup> and kidney.<sup>179</sup> Additionally, its loss promotes and can even cause replication stress and stress-induced genomic alterations without activation of the DNA damage response pathway.<sup>173,180,181</sup> Fhit-deficiency is also associated with insufficient deoxythymidine triphosphate (dTTP) levels which are crucial for efficient DNA synthesis and preservation of genome stability.<sup>180</sup> Overall, Fhit deletion might be the initiator of further cancer development.<sup>170</sup> Re-expression of Fhit, however, reduces DNA damage and genomic instability and might confirm its role as genomic caretaker.<sup>182</sup>

## 2.3 Tumor Suppressor

In addition to its role as genome caretaker, Fhit is also investigated as tumor suppressor protein. In this context, complex formation between Fhit and Ap<sub>3</sub>A is crucial and marks the starting point of a signal transduction pathway towards apoptosis (**Figure 14**). Non-cancerous cells, exposed to genotoxic stress, can counteract by producing increased amounts of Ap<sub>3</sub>A and triggering apoptosis before malignancies are developed.<sup>67</sup> Tumor cells having lost Fhit expression, however, have also lost sensitivity to damage signals and cannot proceed towards cell cycle arrest or cell death. Hence, cell proliferation is inevitable. On the other side, restoration of Fhit expression by viral vectors in cancer cells significantly reduces the proliferation rate or induces apoptosis.<sup>183,184</sup> Further studies revealed that Fhit also has influence on the epithelial-to-mesenchymal transition of tumors thereby reducing their invasiveness and metastatic potential.<sup>185,186</sup> Thus, Fhit is believed to be a tumor suppressor protein.

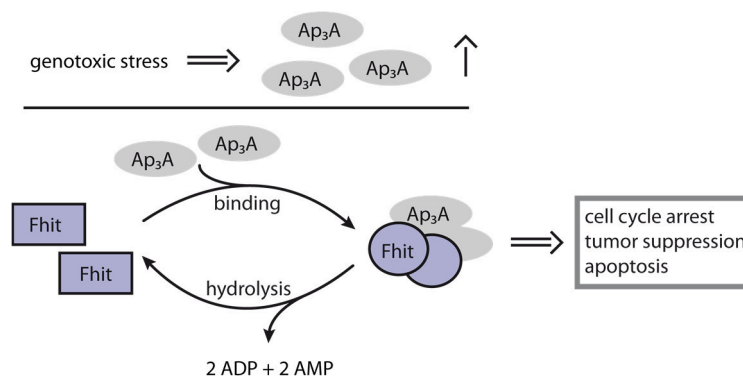


**Figure 14:** Overview on Ap<sub>3</sub>A metabolism. Increased levels of Ap<sub>3</sub>A, induced by cellular stress, are down-regulated by Fhit which cleaves Ap<sub>3</sub>A into AMP and ADP. Upon complex formation, Fhit's tumor suppressor functionality is activated. Figure adapted from<sup>189</sup>.

The signaling functionality in complex with Ap<sub>3</sub>A is not related to enzymatic activity. Mutants altering the central histidine of Fhit like H96N still allow substrate binding but prevent Ap<sub>3</sub>A hydrolysis and do not hinder apoptotic signaling.<sup>165,187,188</sup> Crucial, however, is binding to Ap<sub>3</sub>A.<sup>188</sup> Due to Fhit's enzymatic cleavage of Ap<sub>3</sub>A the complex's lifetime and hence, the signaling event, are only short-lived.

## 2.4 DNA Damage and Stress Response

Increased numbers of spontaneous DNA double-strand breaks (DSBs) have been detected in cells with decreased Fhit expression. Usually, DNA damage activates checkpoints to slow down the cell cycle promoting DNA repair and preserve genomic integrity.<sup>190-193</sup> In case of severe damage, also apoptosis can be induced. DNA damage due to Fhit-deficiency, however, does not induce cell cycle control. Hence, DNA is not repaired and further contributes to genome instability.<sup>167,180,194,195</sup> Moreover, it has been shown that Fhit  $-/-$  and Fhit  $+/-$  cell lines derived from mice have a higher probability of developing spontaneous tumors compared to Fhit  $+/+$  cells.<sup>196</sup> Experiments with Fhit mutant Y114A, a variant with very poor substrate-binding,<sup>188,197</sup> showed that it was unable to suppress spontaneous DSBs, whereas the H96N mutant without hydrolytic activity but good substrate-binding reduced DSBs to the same extent as did wild-type Fhit.<sup>182</sup>

After DNA damage, the checkpoint kinases ATR and CHK1 are some of the most important signal transducer regulating damage checkpoints.<sup>198-202</sup> Prevention of genomic defects by Fhit is dependent on CHK1 activity and might involve Fhit phosphorylation by CHK1 leading to promotion of DNA replication (**Figure 15**).<sup>182</sup>

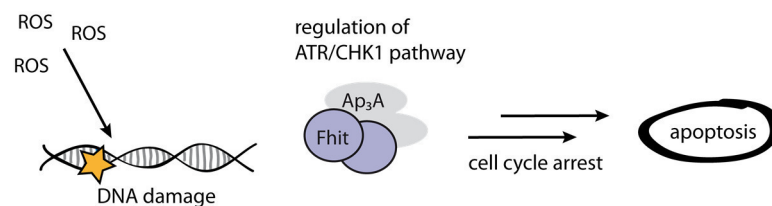


**Figure 15:** Involvement of Fhit and Ap<sub>3</sub>A in DNA damage. DNA damage by ROS leads to interaction of Fhit with the regulation of the ATR/CHK1 pathway and can trigger cell cycle arrest or apoptosis.

Fhit-deficient cells also show a stronger checkpoint-response than Fhit  $+/+$  cells when stress is induced by infrared (IR) irradiation. This causes a higher radioresistance of Fhit  $-/-$  cells, in which DNA damage already accumulates due to Fhit-deficiency. Hence, loss of Fhit expression leads to enhanced DNA repair and to increased survival of cells with a higher chance of mutations.<sup>203</sup> Likewise, exposure to UV irradiation or the DNA crosslinking agent mitomycin C lead to reduced induction of apoptosis.<sup>204</sup>

In response to oxidative stress, Fhit is transported into the mitochondria by the chaperone 60 kDa heat shock protein (Hsp60) where it stabilizes Ferredoxin reductase (Fd<sub>ox</sub>r). Thus, an increased reactive oxygen species (ROS) production is enabled by rising intramitochondrial Ca<sup>2+</sup> concentrations and apoptosis is triggered (**Figure 16**).<sup>205,206</sup> Fhit-deficient cells survive oxidative damage and may pass their DNA damage on to the next generation.

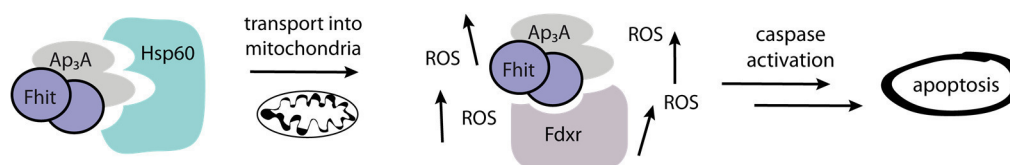


**Figure 16:** Transport of Fhit into mitochondria by Hsp60. Interaction of Fhit with Fd<sub>ox</sub>r leads to increase of ROS and Ca<sup>2+</sup> uptake and apoptosis by caspase activation is triggered.

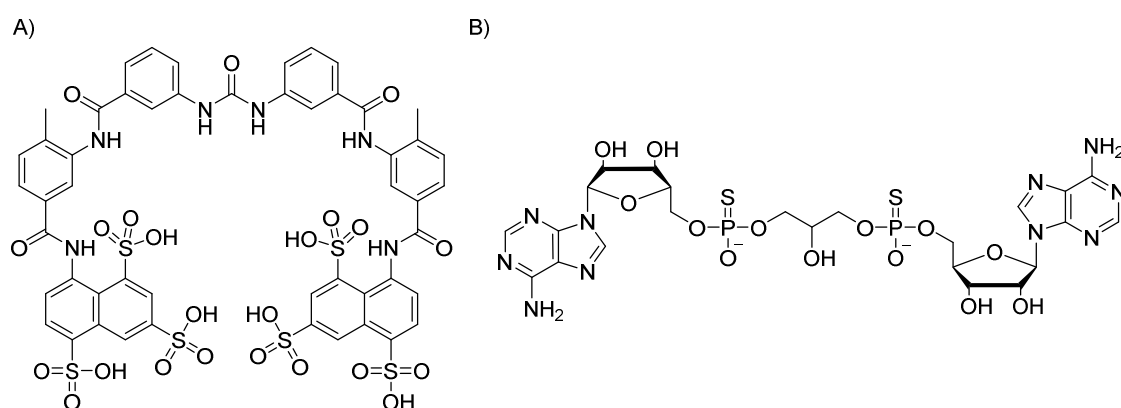
## 2.5 Fhit Interaction Partners

With respect to its functions as a genome caretaker and tumor suppressor, interacting and modifying proteins are important to further understand Fhit's physiological role. A binding protein of the Fhit-Ap<sub>3</sub>A complex is the small ubiquitin-related modifier (SUMO)-conjugated enzyme (Ubc9).<sup>207</sup> Binding of Ubc9 prolongs the lifetime of the complex and hence, apoptotic signaling.<sup>165,188,208</sup> Furthermore, Fhit is phosphorylated at Y114 by Src family protein kinases *in vitro* and *in vivo*.<sup>209</sup> Y114 seems critical for active signaling of the Fhit-Ap<sub>3</sub>A complex towards caspase-dependent apoptosis by also favoring the complex's lifetime.<sup>210</sup> However, it also might be a signal for downregulation of Fhit expression by activation of the epidermal growth factor receptor (EGFR)-Src signaling pathway.<sup>210</sup>

As described in 2.4, Fhit has been shown in several studies to interact with Fd<sub>ox</sub>r and Hsp60, inducing apoptosis upon oxidative stress.<sup>197</sup> Fhit furthermore interacts with tubulin, regardless of its enzymatic activity status.<sup>211,212</sup> An interesting finding was the relationship between Fhit and beta-catenin which represses the transcription of target genes like cyclin D1, MMP-14 and survivin.<sup>213</sup> Furthermore, Fhit has influence on the regulation of proteins in the Ras/Rho GTPase molecular switch, however, direct interactions are unknown.<sup>185</sup> With respect to DNA damage, Fhit regulates thymidine kinase 1 (TK1) expression.<sup>180,214</sup> This kinase is involved in the synthesis of dTTP whose level is largely reduced upon loss of Fhit followed by a decrease in TK1 expression.<sup>180,215</sup> Restoration of Fhit is ensued by re-expression of TK1.<sup>214</sup> A cooperation of Fhit with Nitrilase 1 (Nit1) is proposed based on the Rosetta Stone hypothesis. In flies and worms Nit1 and Fhit form a fusion protein and have tumor suppressive functionality.<sup>216</sup> Evidence for interactions in mammals, however, has not been presented so far.

## 2.6 Fhit Inhibitors

Small molecules that inhibit the hydrolytic activity of Fhit are valuable tools to further elucidate and understand its tumor suppressor activity. Up to now only a few compounds have been presented as Fhit inhibitors including the small molecule Suramin (see **Scheme 10A**)<sup>217</sup> and non-cleavable Ap<sub>n</sub>A analogues,<sup>159,165,218</sup> next to the transition metal ions Cu<sup>2+</sup> and Zn<sup>2+</sup>.<sup>219</sup> The metal ions, however, exert a wide range of functions and have many targets within cells. Hence, Fhit is inhibited rather unspecifically. Similarly, Suramin also targets various classes of enzymes.<sup>220,221</sup> Additionally, IC<sub>50</sub> values have been measured in the micromolar rather than the nanomolar range, which is insufficient for intense studies.<sup>222</sup> Besides contributing to a better understanding of Fhit, inhibitors might have anti-cancer activity if the complex formed by inhibitor and protein has pro-apoptotic signaling function itself or if the inhibitor stabilizes the signaling Fhit-Ap<sub>3</sub>A complex. It was reported that a directly competing, non-hydrolyzable Ap<sub>n</sub>A analogue induces Fhit-dependent and caspase-directed apoptosis, which supports this hypothesis (**Scheme 10B**).<sup>223</sup>



**Scheme 10:** Structures of known Fhit inhibitors Suramin (A) and a non-hydrolyzable Ap<sub>4</sub>A analog (B).

## 2.7 Förster Resonance Energy Transfer (FRET)

The non-radiative transfer of energy between a donor chromophore and an acceptor chromophore in close proximity is described by the Förster resonance energy transfer (FRET), discovered in 1948.<sup>224</sup> The energy transfer is achieved by dipole-dipole coupling when three requirements are fulfilled: the spectral overlap of donor emission and acceptor absorption must account for at least 30%,<sup>225</sup> the maximum spatial distance between the two chromophores must not exceed 10 nm and the relative orientation of the two dipole moments must be appropriate to one another. Additionally, a high quantum yield of the donor is advantageous and simplifies the measurement due to an increased FRET signal.<sup>226</sup> The Jablonski diagram for FRET between two fluorophores is displayed in **Figure 17A**. Instead of fluorescence emission upon excitation of the donor, the energy is transferred to the energy-ground state of the acceptor. As a

consequence fluorescence emission of the acceptor is detected, distinctly shifted to higher wavelengths (**Figure 17B**). FRET is not limited to fluorescent chromophores, but can also occur in presence of a quencher or be expanded to phosphorescence.<sup>227</sup> FRET is directly coupled to the distance  $R$  between donor and acceptor which makes it extremely sensitive to small changes in the spatial configuration. The FRET efficiency  $E$  describes the ratio of the amount of donor excitation events that result in FRET to the total number of donor excitation events.  $E$  is linked to  $R$  via the following formula:

$$E = \frac{R_0^6}{R^6 + R_0^6}$$

The Förster distance  $R_0$  represents the distance at which the FRET efficiency is 0.5 (see **Figure 17C**).<sup>227</sup> FRET can occur at large biomolecules as well as small molecules, intra- and intermolecular (see **Figure 17D**).<sup>228</sup> By choice of different donor-acceptor pairs the ideally suited FRET pair for every biochemical application can be identified. Recently, a doubly modified Ap<sub>3</sub>A analog was synthesized that was equipped with the Sulfo-Cy3/Sulfo-Cy5 FRET pair and utilized to follow cleavage by Fhit.<sup>222</sup> Hence, this molecule should be ideally suited to study inhibitors of the enzymatic activity of Fhit.

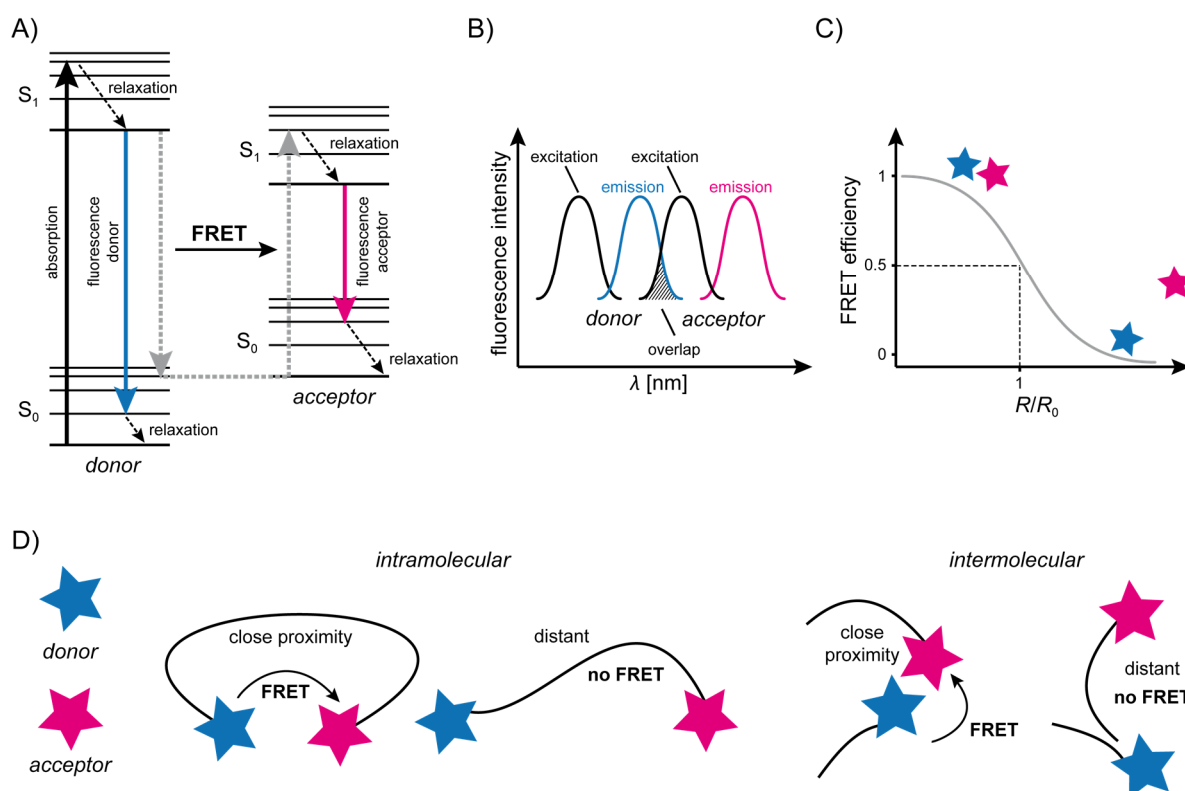


**Figure 17:** Principles of FRET. A) Jablonski diagram to describe FRET when donor and acceptor are both fluorophores. B) Excitation and emission spectra of donor and acceptor. The overlap of donor emission and acceptor excitation is crucial. C) FRET efficiency as a function of  $R/R_0$ . D) FRET can occur intramolecularly or intermolecularly when donor and acceptor are in close proximity. A, B, C adapted from<sup>227</sup>



## II Aim of the Thesis

The family of adenosine nucleotides with its multiple members and various functions *in vivo* is present in the major part of biological processes in most organisms. The most abundant and long-known member of this family is ATP as the main energy supplier in life. Diadenosine polyphosphates represent another part of these nucleotides, whereas they are less familiar. The  $\text{Ap}_n\text{A}$  family consists of seven members, among which  $\text{Ap}_3\text{A}$  and  $\text{Ap}_4\text{A}$  have been the most studied representatives since their discovery over 50 years ago. Although many studies targeted their physiological role, to date, it is still widely unclear. Stress conditions were identified as one source for elevated intracellular levels, however, effects were identified in several cellular pathways and clear functionalities for the particular  $\text{Ap}_n\text{As}$  yet need to be determined.

The investigation of a small molecule's interactome can deliver a great amount of information regarding its processing and involvement in cellular pathways. In the past, several studies have been performed on identifying interacting proteins of  $\text{Ap}_4\text{A}$ , other  $\text{Ap}_n\text{A}$  family members, however, have not been investigated so far.<sup>33,57-59</sup> For this reason, one objective of this thesis was the development and implementation of an ABPP procedure to identify new interaction partners of  $\text{Ap}_3\text{A}$  in human cell lysates. Modern proteomics with LC-MS/MS analyses and software-assisted evaluation of the obtained results should deliver the interactome that might point towards a physiological role of  $\text{Ap}_3\text{A}$ .

Another strategy to obtain further knowledge about the cellular role of  $\text{Ap}_3\text{A}$  involved the analysis of the so far only known and studied interaction partner of  $\text{Ap}_3\text{A}$ , the Fhit protein. Detailed mechanisms of its tumor suppressor functionality in complex with  $\text{Ap}_3\text{A}$  are still obscure, however, Fhit's properties of hydrolyzing  $\text{Ap}_3\text{A}$  appear to stop signaling towards apoptosis. Therefore, the discovery of small molecule inhibitors of the enzymatic activity of Fhit would provide valuable tools in obtaining further information about the significance of  $\text{Ap}_3\text{A}$  in this context and the tumor suppressive character of Fhit. Hence, a FRET-based high-throughput screening should be conducted to identify potential inhibitors of Fhit's hydrolytic activity. By evaluation of the structure-activity relationship, derivatives should be synthesized in order to obtain improved compounds. A detailed analysis in a dose-response manner, as well as a characterization of the cytotoxic potential for future intracellular applications should complete the picture.

Altogether, the development of various chemical tools towards the investigation of  $\text{Ap}_3\text{A}$  should help to further elucidate its cellular functionalities.



### III Results and Discussion

#### 1 Interactome of Ap<sub>3</sub>A

To date, little details are known about the physiological role of Ap<sub>3</sub>A and its family members. Although involved in many biological processes, exact mechanisms still remain to be discovered. In order to elucidate signal transduction pathways, the interactome of the molecule of interest is especially important. Despite several attempts to analyze interacting partners of Ap<sub>4</sub>A, Ap<sub>3</sub>A has never been the object of interest in this respect so far.<sup>33,57-59</sup> Thus, the main focus in this part of the thesis lies in the design and synthesis of a suitable Ap<sub>3</sub>A probe to develop and implement an ABPP procedure for the analysis of the interaction network of Ap<sub>3</sub>A.

##### 1.1 Design of ABP Probe A

For the identification of human proteins interacting with Ap<sub>3</sub>A an ABP had to be developed that matched several criteria. First of all, Ap<sub>3</sub>A had to remain hydrolytically stable against specific or unspecific enzymes in cellular lysates under ABPP conditions. This was achieved by introducing a sulfur atom for one of the oxygens at the  $\alpha$ -phosphate group in addition to a methylene bridge between the  $\beta$ - and  $\gamma$ -phosphate.<sup>159</sup> In this way, the  $\alpha$ - $\beta$ -phosphate as well as the  $\beta$ - $\gamma$ -phosphate bond were protected from hydrolysis and the stable Ap<sub>3</sub>A could be used to capture binding or processing proteins. For this purpose, the Ap<sub>3</sub>A probe further required an affinity handle to separate interacting partners from unbound cell lysate. Thus, the high affinity between biotin and streptavidin<sup>79</sup> was chosen and a biotin functionality was implemented in the ABPP procedure. Since the biotin moiety with a flexible linker is a rather space-demanding group an alkyne handle was incorporated in the ABP to keep the molecule as small as possible during the incubation with cell lysates. Later, a CuAAC reaction could be performed to introduce biotin as affinity tag. To further improve the Ap<sub>3</sub>A probe, a photoaffinity functionality was introduced to link the probe covalently to interacting proteins. This step simplifies washing procedures during ABPP since more stringent conditions can be applied to prevent extensive unspecific binding. The easiest and previously reported way to implement a photoaffinity tag was to attach an azide in position C8 of one adenosine.<sup>99,102</sup> Upon irradiation with UV light at 366 nm a nitrene is formed that can initiate different types of reactions with a partner in close proximity. Among these are C-H or N-H insertions and ring expansions of the nitrene into the aryl ring, followed by reaction with primary amines.<sup>96</sup>

Several possibilities were available to attach the modifications at the Ap<sub>3</sub>A molecule. Therefore, the only known crystal structure of an Ap<sub>3</sub>A analogue in complex with a protein was chosen as guideline. The crystal structure of a non-hydrolyzable Ap<sub>3</sub>A analogue in complex with the Fhit protein (see **Figure 18A**)<sup>165</sup> revealed sufficient space in position N6 at one adenosine since it is located outside of Fhit. Hence, the alkyne-handle was chosen to be incorporated there, making the probe accessible for CuAAC. Furthermore, modifications in position N6 were shown to be tolerated as substrates by Fhit.<sup>222</sup> Position C8 at the  $\gamma$ -phosphate-linked adenosine seemed to be ideally suited to host the relatively small azido group since there is only a small distance between C8 and the protein where photoinduced crosslinking can proceed. Overall, the ABP probe A should be synthesized as a non-hydrolyzable molecule with tags for photoaffinity crosslinking and a CuAAC handle. The molecular structure of probe A is depicted in **Figure 18B**.

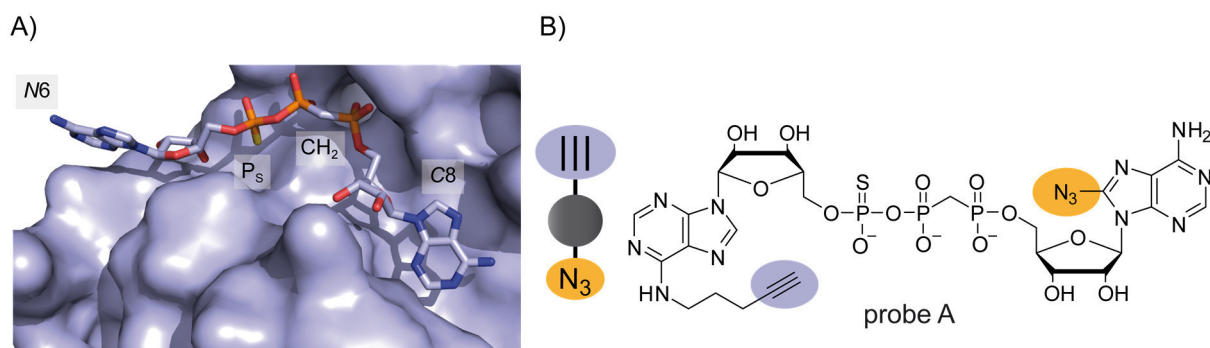
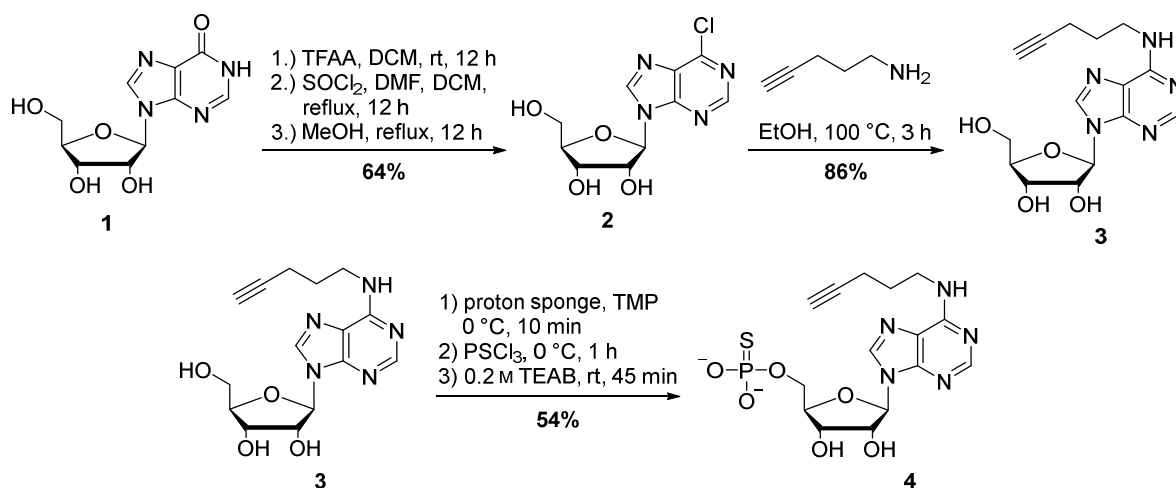


**Figure 18:** Design of probe A. Crystal structure of Fhit in complex with a non-hydrolyzable Ap<sub>3</sub>A analogue (A, PDB 1FHI)<sup>165</sup> and molecular structure of probe A (B).

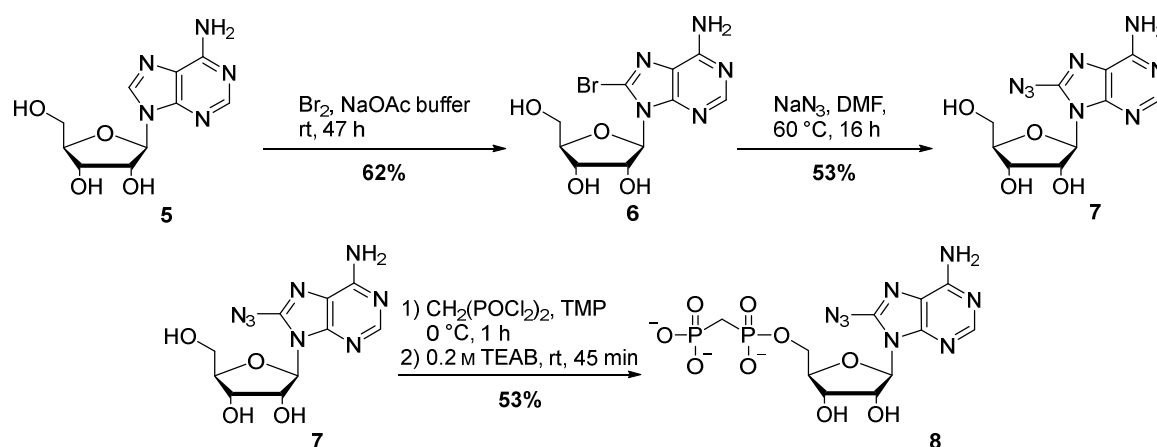
## 1.2 Synthesis of Probe A

The synthesis of probe A was started from commercially available inosine (**1**) treated with thionyl chloride after *in situ* TFA protection of the primary hydroxyl groups. The resulting compound **2** was obtained by deprotection with MeOH.<sup>229</sup> In a reaction with 4-pentynyl amine in EtOH N6-(pent-4-yn-1-yl)adenosine (**3**) was formed.<sup>230</sup> Phosphorylation with PSCl<sub>3</sub> in trimethylphosphate (TMP) with proton sponge gave compound **4** in analogy to the literature,<sup>231</sup> one of the building blocks towards non-hydrolyzable Ap<sub>3</sub>A. All reactions containing phosphates had to be purified by anion-exchange chromatography, RP-HPLC and several steps of lyophilization with water. The synthesis scheme for compound **4** is depicted in **Scheme 11**.



**Scheme 11:** Synthesis scheme for the preparation of N<sup>6</sup>-(pent-4-yn-1-yl)adenosine 5'-thiomonophosphate (4).

The synthesis of the second building block (**8**, see **Scheme 12**) was started by the electrophilic aromatic substitution of adenosine (**5**) with bromine in NaOAc buffer to give compound **6**.<sup>232</sup> Subsequent nucleophilic substitution with NaN<sub>3</sub> in DMF in position C8 yielded 8-azidoadenosine (**7**).<sup>233</sup> Compound **7** was further diphosphorylated with methylene bisphosphonic dichloride in TMP to give **8** in analogy to a reported procedure.<sup>234</sup> This reaction was monitored by analytical HPLC to obtain more information about the reaction time and progress (see **Figure 19**). After 3.5 h the reaction was stopped since a stable relation between product and side product was reached and the residual starting material was minimal. In the beginning of the reaction also the dinucleotide was formed which, however, was degraded in the further reaction progress and absent after 1.5 h.



**Scheme 12:** Synthesis scheme for the preparation of 8-azidoadenosine 5'-methylene bisphosphonate (8).

The coupling as the final step in the synthesis of probe A was conducted similarly to a literature procedure introduced by Blackburn *et al.*<sup>159</sup> In their synthesis they describe the activation of the non-modified adenosine 5'-thiomonophosphate with diphenylphosphoryl chloride and the subsequent addition of a non-modified adenosine 5'-methylene bisphosphonate. Hence,

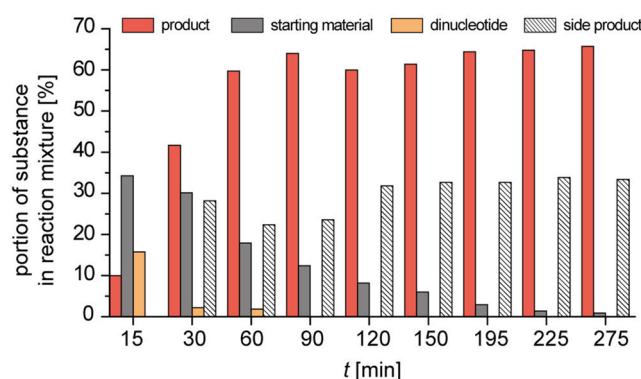
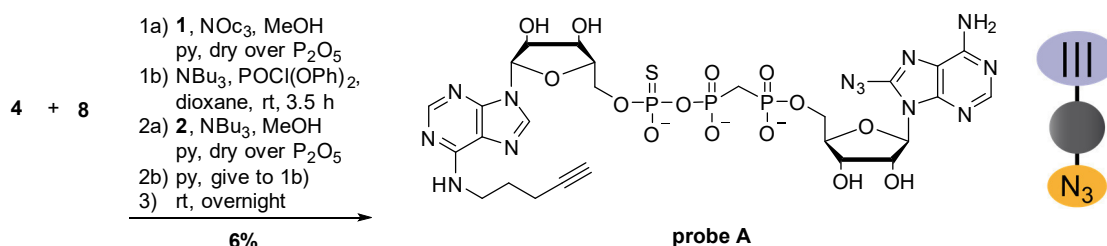


**Figure 19:** Result of an analytical HPLC analysis of the methylene bisphosphorylation of 8-azidoadenosine over time.

compound **4** was activated with diphenylphosphoryl chloride in dioxane and coupled to **8** in pyridine to give the modified non-hydrolyzable Ap<sub>3</sub>A probe A. The product was first obtained as a mixture of diastereoisomers. Further RP-HPLC, however, led to the separation of two pure products as their triethyl ammonium salts. The synthesis scheme is depicted in **Scheme 13**. A coupling method, previously described by Mohamady and Taylor for the synthesis of hydrolyzable Ap<sub>3</sub>A, was conducted to compare the yields and synthesis effort.<sup>139</sup> Here, the methylene bisphosphonate was activated by *N*-methylimidazole, however, coupling to the thiophosphate was not successful.



**Scheme 13:** Synthesis of probe A. Coupling of *N*6-(pent-4-yn-1-yl)adenosine 5'-thiomonophosphate (**4**) and 8-azidoadenosine 5'-methylene bisphosphonate (**8**) yields the modified Ap<sub>3</sub>A analogue probe A.

### 1.3 Characterization of Probe A

The next step after the successful synthesis was the confirmation of the newly developed non-hydrolyzable Ap<sub>3</sub>A to be non-degradable by specific and non-specific enzymes. Therefore, an analytical HPLC assay was set up in which probe A was incubated with either the specific Ap<sub>3</sub>A hydrolase Fhit or the unspecific snake venom phosphodiesterase (SVPD).<sup>235</sup> The same experiments with natural Ap<sub>3</sub>A served as a positive control that all employed enzymes were indeed active. To perform the assay, first, recombinant Fhit was expressed and purified as described in the literature via Ni-NTA beads.<sup>236</sup> In the analytical assay (see **Figure 20**), natural Ap<sub>3</sub>A exhibited a retention time of ~14 min when incubated without enzyme. Upon addition of Fhit natural Ap<sub>3</sub>A was cleaved into AMP (12 min) and ADP (13 min), whereas it was degraded to AMP (12 min) when the commercially available non-specific phosphodiesterase SVPD was

employed. In the experiments with probe A, the retention time of 20 min was obtained when no enzyme was present. Incubation with either Fhit or SVPD did not lead to any change in retention time. Hence, the experiments confirmed that probe A was indeed stable towards hydrolysis by specific and non-specific enzymes and could therefore be applied in the envisaged ABPP assays.

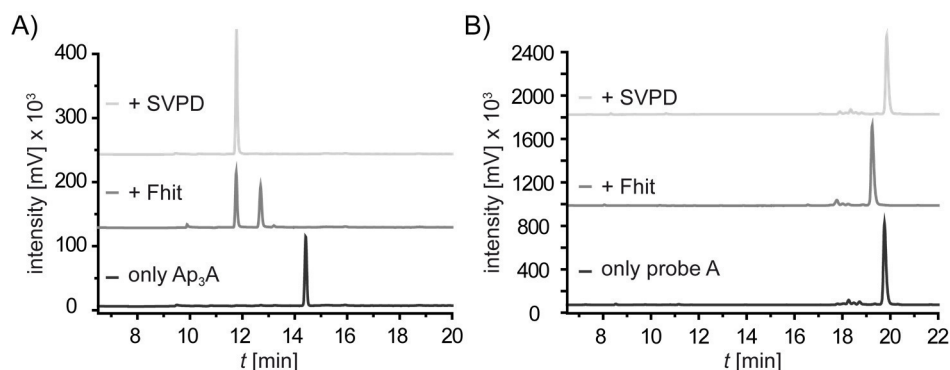


**Figure 20:** Characterization of probe A. Result of the analytical HPLC analysis of natural Ap<sub>3</sub>A (A) and probe A (B), incubated with Fhit, SVPD or without enzyme.

Next, it was studied if probe A was still able to bind to the tumor suppressor Fhit. Therefore, probe A's ability to inhibit cleavage of a hydrolyzable Ap<sub>3</sub>A substrate by addressing the same binding pocket of Fhit should be tested. A hydrolyzable doubly labeled Ap<sub>3</sub>A analogue was previously synthesized in our group by Stephan M. Hacker and was able to undergo FRET (see **Figure 21A**).<sup>222</sup> Upon cleavage by Fhit the fluorescence characteristics change drastically when the interaction between the donor dye Sulfo-Cy3 and the acceptor dye Sulfo-Cy5 is interrupted. In a fluorescence-based assay at 535 nm excitation the emission of the donor Sulfo-Cy3 was measured at 590 nm which increased significantly upon separation from the acceptor dye. Fhit was pre-incubated with increasing amounts of probe A, FRET-Ap<sub>3</sub>A was added and time points

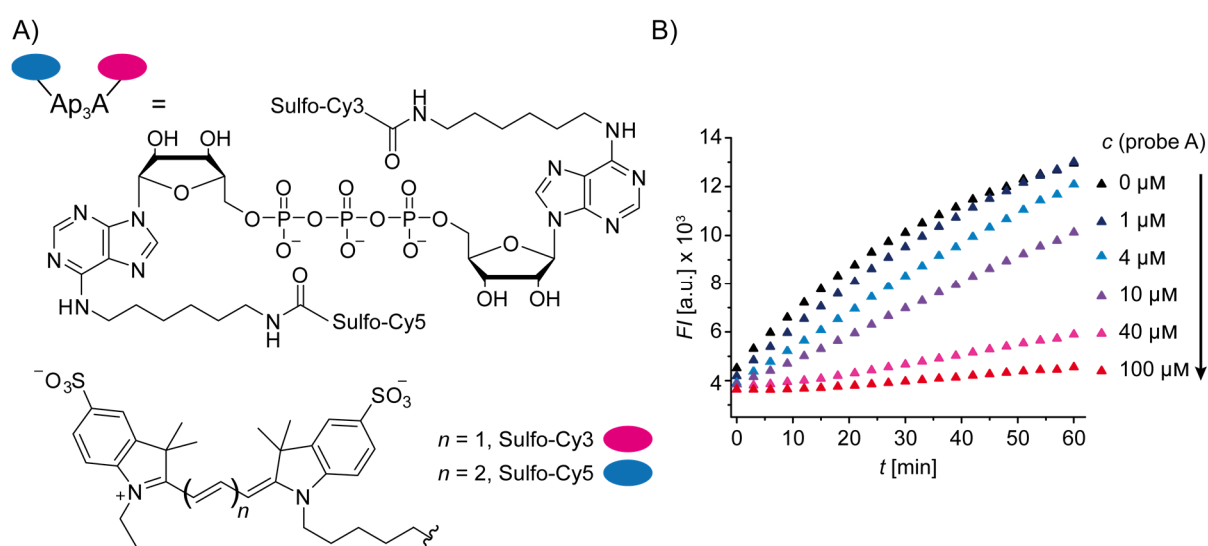


**Figure 21:** FRET-Ap<sub>3</sub>A. A) Doubly fluorescently labeled hydrolyzable Ap<sub>3</sub>A analogue (FRET-Ap<sub>3</sub>A). B) Plot of the fluorescence intensity over time for the hydrolysis of FRET-Ap<sub>3</sub>A (10  $\mu$ M) by Fhit with increasing amounts of probe A.

were taken every 3 min over 60 min. As is depicted in **Figure 21B** cleavage was disturbed by the non-hydrolyzable Ap<sub>3</sub>A and even completely inhibited when 100  $\mu$ M of probe A were applied. This implied that probe A was a competitive inhibitor for Fhit.

#### 1.4 Photoaffinity Labeling of Fhit by Probe A with Subsequent CuAAC

In a follow-up experiment it was studied if covalent substrate binding could be achieved via photocrosslinking between Fhit and probe A when they were exposed to long-wave UV light. Subsequent CuAAC should be performed to install the biotin moiety in the Ap<sub>3</sub>A-protein complex. As a first test, N<sub>3</sub>-Cy5 (**Figure 22A**) was used as a reagent to investigate if CuAAC could be implemented at all with THPTA (**Figure 22B**) as ligand and the Cu<sup>I</sup> salt tetrakis-(acetonitrile)copper(I)-tetrafluoroborate (**Figure 22C**). A schematic overview of the proposed assay is given in **Figure 22D**. The fluorescent properties of N<sub>3</sub>-Cy5 were a great advantage since a fluorescent read-out of the SDS-PAGE analysis could be performed whose sensitivity is much higher than of a Coomassie-stained gel.<sup>237</sup> Therefore, less material could be used for the respective test reactions.

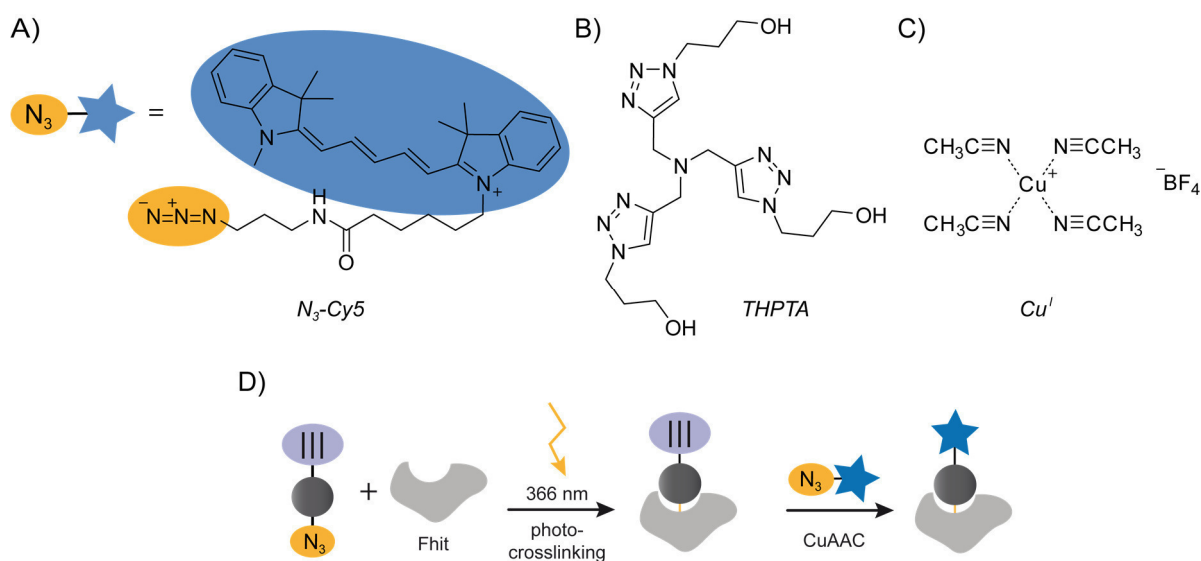


**Figure 22:** Photoaffinity labeling of Fhit: Molecular structures and principle. Molecular structures of the CuAAC reagents N<sub>3</sub>-Cy5 (A), THPTA (B) and the utilized Cu<sup>I</sup> salt (C). D) Schematic overview on the photocrosslinking reaction of probe A to Fhit with subsequent CuAAC to N<sub>3</sub>-Cy5.

Upon irradiation for 60 min at 366 nm a new band, additional to and above Fhit, was detected in the chemiluminescent western blot (WB) against a Fhit antibody (**Figure 23A**). This new band could be assigned to the covalent complex of Fhit and probe A. When a subsequent CuAAC was performed with N<sub>3</sub>-Cy5, Cu<sup>I</sup> and THPTA after the photoreaction an upwards shift of the new band was observed, caused by the complex of Fhit, probe A and the Cy5 dye. In the fluorescent read-out of the gel of a similar experiment a fluorescent band could be detected at around 20 kDa when the sample was irradiated for 30 min and an even stronger band when

the UV exposure lasted 60 min (**Figure 23B**). As a fluorescent band at this height of the gel could only be induced by the complex of Fhit, probe A and the Cy5 dye this first experiment proved to be successful. Additionally, neither a fluorescent band nor a shift on the WB was observed when no UV light had been used (lane 1).

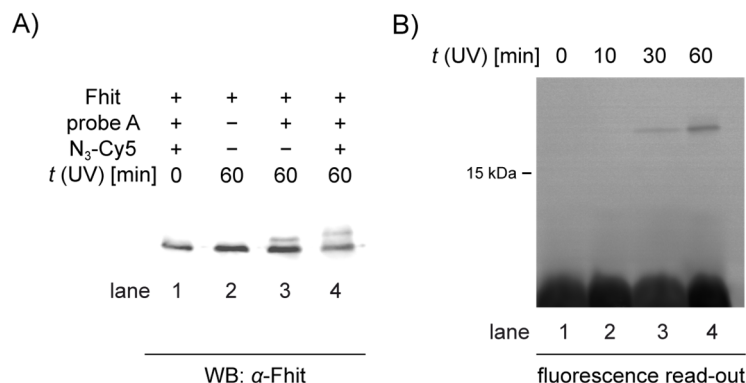


**Figure 23:** Photoaffinity labeling of Fhit. A)  $\alpha$ -Fhit WB analysis of a photoreaction of Fhit and probe A with UV light at 366 nm and subsequent CuAAC reaction to N<sub>3</sub>-Cy5. B) Fluorescent read-out of a photoreaction of Fhit and probe A with UV light at 366 nm and subsequent CuAAC reaction to N<sub>3</sub>-Cy5 with different lengths of UV irradiation.

The next step was to examine whether labeling of Fhit by probe A was specific or if proteins without known binding sites for nucleotides could be labeled as well. For this purpose, bovine serum albumin (BSA) was used as a test protein and negative control in a series of experiments with photoreaction, subsequent CuAAC with N<sub>3</sub>-Cy5 and a fluorescent read-out of the SDS-PAGE analysis. Several conditions with different concentrations of probe A, Fhit and BSA were studied. As a result, BSA was labeled by probe A in almost all conditions even when mixed with Fhit (**Figure 24A**). Solely one condition (lane 1) was found in that only Fhit was labeled in a mixture of Fhit and BSA. From the crystal structure it is known that Ap<sub>3</sub>A is bound with its

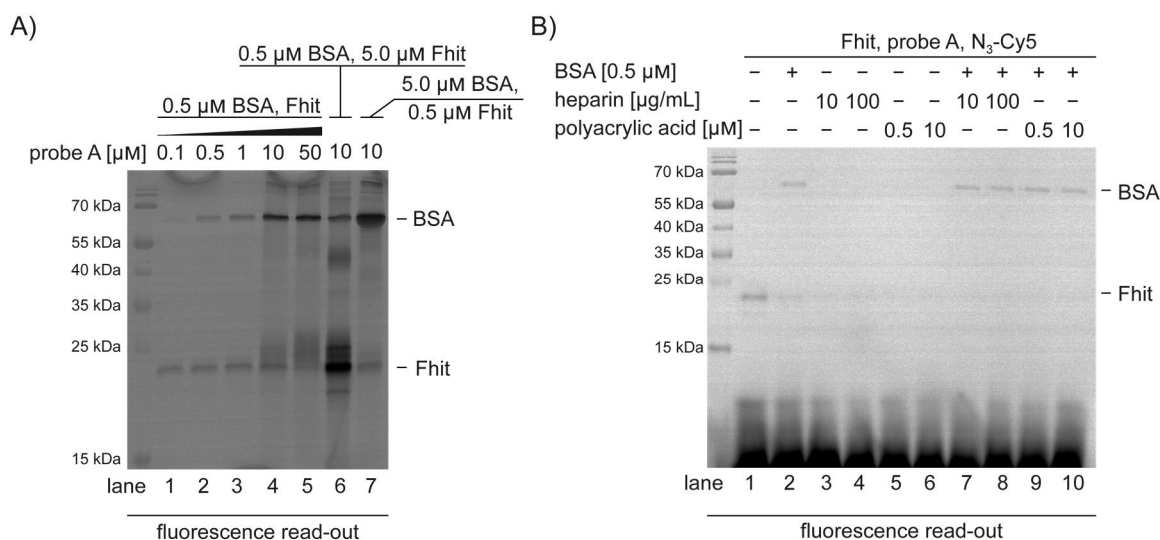


**Figure 24:** Photoaffinity labeling of Fhit and BSA. A) Fluorescent read-out of photoreactions of Fhit, BSA and probe A and subsequent CuAAC with N<sub>3</sub>-Cy5 to identify if probe A is specific for Fhit. B) Fluorescent read-out of photoreactions of Fhit, BSA and probe A with addition of heparin or polyacrylic acid and subsequent CuAAC with N<sub>3</sub>-Cy5 to investigate whether binding to Fhit or BSA was disturbed.

phosphate chain to the highly positively charged surface of Fhit. To analyze whether the interaction with BSA is based on a solely ionic interaction between the negative phosphate chain of probe A and some unspecific side chains of BSA it was investigated if negatively charged heparin and polyacrylic acid were able to disrupt binding to either Fhit or BSA. Both reagents were applied in different amounts and prohibited labeling of probe A to Fhit completely while labeling of BSA was not affected at all (**Figure 24B**). This implied that some other interaction might be responsible for the fluorescent band on the gel in case of BSA.

To further investigate this interaction, it was tested if BSA could be labeled with N<sub>3</sub>-Cy5 in absence of probe A. Indeed, it did not make a difference in the fluorescent read-out if probe A was present or not (**Figure 25A**). Since the fluorescent band on the gel was completely independent from probe A it was concluded that N<sub>3</sub>-Cy5 might not be the perfect choice for

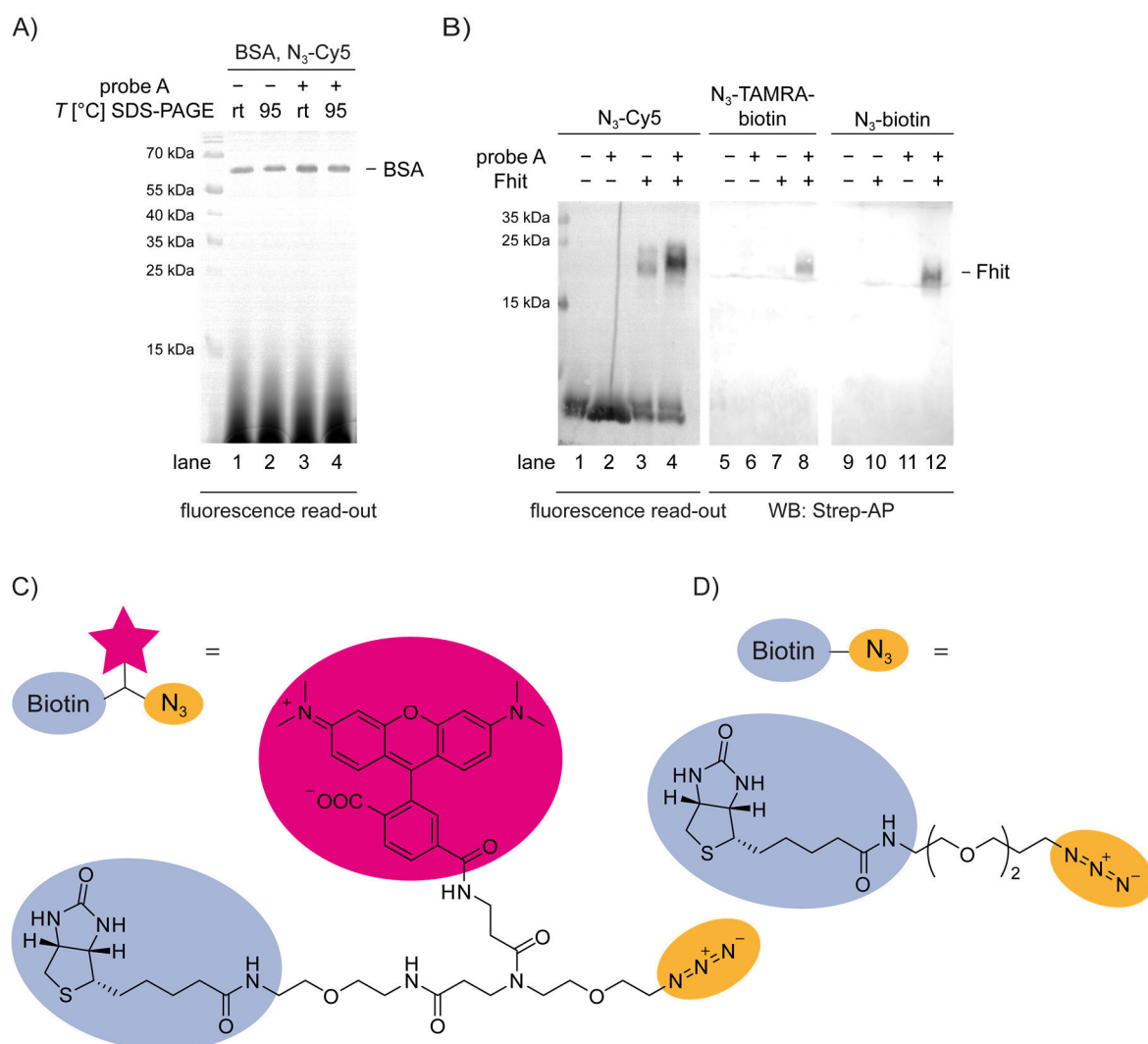


**Figure 25:** Photoaffinity labeling of Fhit with different reagents. A) Fluorescent read-out of a photoreaction of Fhit with subsequent CuAAC to N<sub>3</sub>-Cy5 in presence or absence of probe A. B) Fluorescent read-out and Strep-AP WB analysis of photoreaction of Fhit with subsequent CuAAC reaction to N<sub>3</sub>-Cy5, N<sub>3</sub>-TAMRA-biotin and N<sub>3</sub>-biotin in presence or absence of probe A. C) Molecular structure of N<sub>3</sub>-TAMRA-biotin. D) Molecular structure of N<sub>3</sub>-biotin.

labeling Fhit and investigating the reaction conditions. Therefore, it was tested whether N<sub>3</sub>-Cy5 or other azides in presence or absence of probe A were able to label Fhit under the photoaffinity labeling and CuAAC conditions. In compliance with the results obtained with BSA also Fhit was labeled by N<sub>3</sub>-Cy5 in absence of probe A (**Figure 25B**). These experiments were also conducted with a trifunctional molecule N<sub>3</sub>-TAMRA-biotin (**Figure 25C**), a CuAAC coupling reagent that combined the advantages of a fluorescent read-out via the TAMRA dye with the affinity tag biotin for future ABPP experiments. In a WB analysis via streptavidin-alkaline phosphatase (Strep-AP) it was observed that Fhit was not labeled in absence of probe A (**Figure 25B**, lane 7). The same was proved for the CuAAC reagent N<sub>3</sub>-biotin (**Figure 25D**) that was also not able to bind to Fhit in absence of the Ap<sub>3</sub>A probe. For this reason, N<sub>3</sub>-TAMRA-biotin was utilized instead of N<sub>3</sub>-Cy5 to prevent unsolicited labeling.

Due to the struggles with the CuAAC reaction, a closer look regarding the reaction conditions of the biotinylation with N<sub>3</sub>-TAMRA-biotin was necessary. Therefore, different Cu salts, the previously applied Cu<sup>I</sup> salt and CuSO<sub>4</sub> (Cu<sup>II</sup>), were chosen as well as different Cu<sup>I</sup>-stabilizing

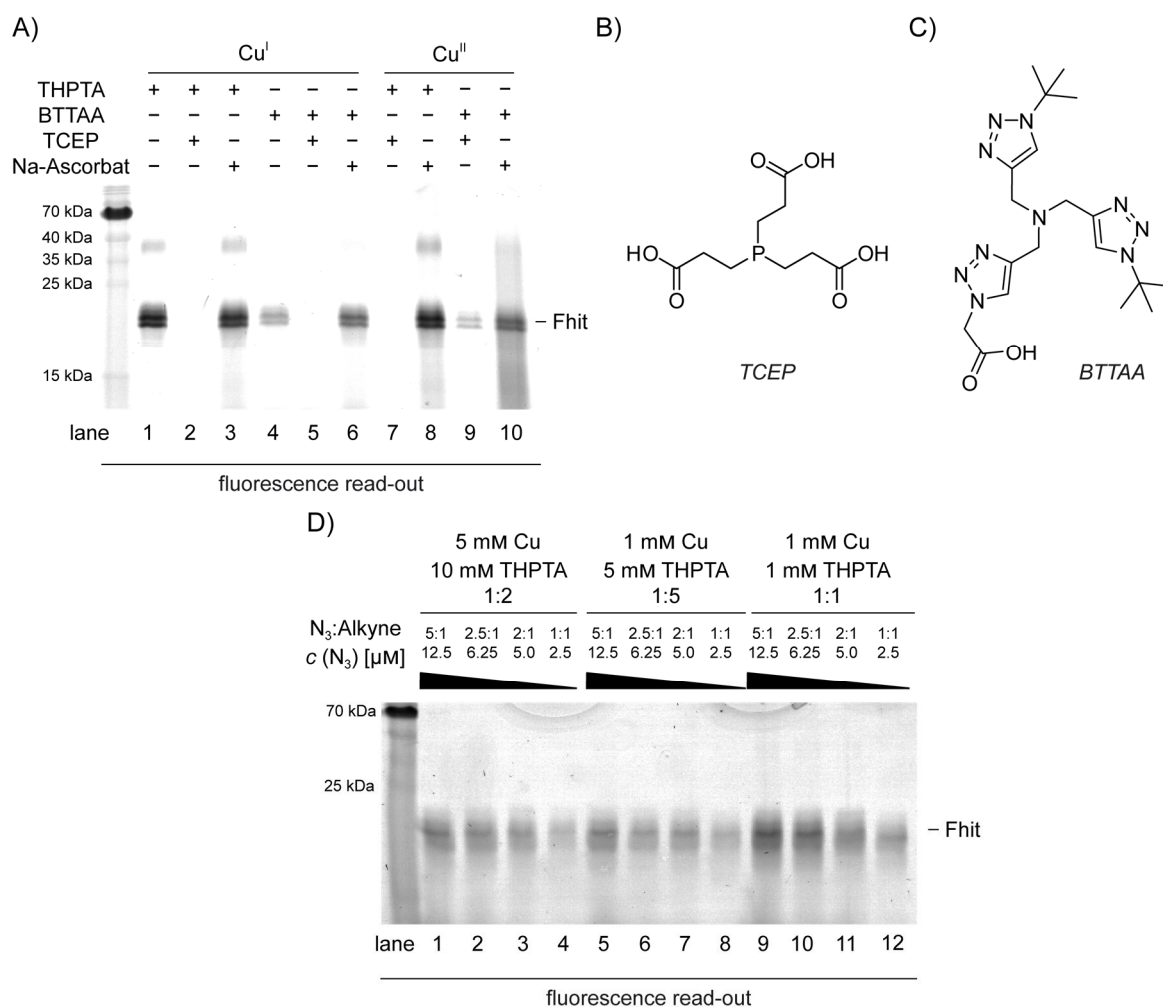


**Figure 26:** Optimization of CuAAC conditions. A) Fluorescent read-out of the photoreaction and subsequent CuAAC of Fhit, probe A and N<sub>3</sub>-TAMRA-biotin when different Cu salts, Cu<sup>I</sup>-stabilizing ligands and reducing agents were applied. B) Molecular structure of TCEP. C) Molecular structure of BTAA. D) Fluorescent read-out of the photoreaction and subsequent CuAAC of Fhit, probe A and N<sub>3</sub>-TAMRA-biotin when different ratios of Cu<sup>I</sup>/THPTA and azide/alkyne were used.

ligands and different reducing agents to either reduce the  $\text{Cu}^{\text{II}}$  salt directly or to reverse the oxidation of the active  $\text{Cu}^{\text{I}}$  salt. The best results were obtained with the  $\text{Cu}^{\text{I}}$  salt and the THPTA ligand without any reducing agents (**Figure 26A**). By contrast, when TCEP (**Figure 26B**) was used as a reductant no reaction occurred at all in any experimental setup. An explanation might be the Cu-binding and azide-reducing properties of phosphines that negatively influence the CuAAC.<sup>238</sup> The addition of sodium ascorbate as reductant improved the reaction with 2-(4-((bis((1-(tert-butyl)-1H-1,2,3-triazol-4-yl)methyl)amino)methyl)-1H-1,2,3-triazol-1-yl)acetic acid (BTAA, **Figure 26C**) as a ligand when  $\text{Cu}^{\text{I}}$  was used, however, it had no enhancing effect with THPTA. Overall, the  $\text{Cu}^{\text{I}}$  reactions worked better than the ones where  $\text{Cu}^{\text{II}}$  was employed. In a further step, the ratios between copper to ligand and azide to alkyne were studied. A ratio of 5:1 of azide to alkyne appeared to give a higher reaction yield than lower rates and the  $\text{Cu}^{\text{I}}$  to THPTA ratio seemed best, when these two components were applied 1:1 (**Figure 26D**).

With these optimized CuAAC conditions the next step was to determine the optimal irradiation time with UV light. Hence, a time scale experiment was conducted in which the sample was irradiated for 120 min. Time points were taken as indicated in **Figure 27**. In a fluorescent read-out of the SDS-PAGE analysis after the photoreaction followed by CuAAC sufficient labeling was already observed after 30 min of irradiation. Longer irradiation times did not lead to additional labeling of the protein. In case of no irradiation, however, a slight fluorescent band occurred as well. In a control reaction with  $\text{N}_3$ -TAMRA-biotin but without  $\text{Cu}^{\text{I}}$  and THPTA no band could be observed after 120 min of irradiation. These results might suggest that probe A was not completely stable under the employed reaction conditions. In this case, a small part of probe A had been already azide-activated beforehand and thus reacted with Fhit, even without UV irradiation. On the other hand, if it is assumed that probe A was stable, the only way to obtain a fluorescent band was unspecific labeling between  $\text{N}_3$ -TAMRA-biotin and Fhit under CuAAC conditions containing  $\text{Cu}^{\text{I}}$ . Taking the previously obtained results with  $\text{N}_3$ -Cy5 into account, the second explanation seemed more reasonable since a reaction of this compound with Fhit in absence of probe A was already documented in presence of the CuAAC reagents.

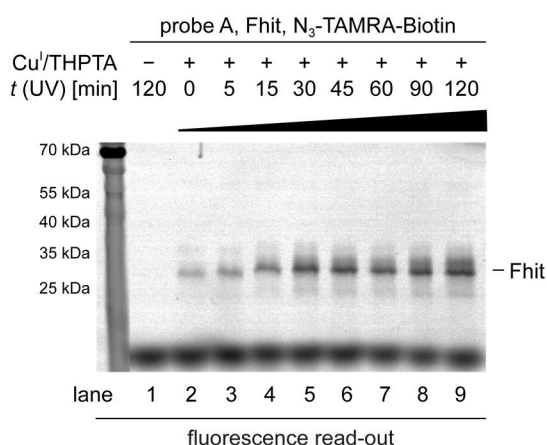


**Figure 27:** Photoaffinity labeling of Fhit over time. Fluorescent read-out of a time scale experiment to identify the optimal UV irradiation time with subsequent CuAAC with  $\text{N}_3$ -TAMRA-biotin.

Despite these drawbacks, the next step was to study if streptavidin bound to the Fhit-Ap<sub>3</sub>A-TAMRA-biotin complex and could be released afterwards. Therefore, the photoreaction and CuAAC was followed by a 10 min incubation step with pure streptavidin. The samples were mixed with SDS loading dye and divided into two parts whereupon half of the samples were boiled at 95 °C for 5 min and the others were loaded onto the gel without previous heating. As a result, streptavidin binding to the biotin-containing complex was successfully achieved as revealed by the upwards shift of the respective band on the gel (**Figure 29A**). The downwards shift of the same band was observed when the sample was heated in loading dye and represents the release of the biotinylated probe A in complex with Fhit from streptavidin.

Since pure streptavidin as an affinity medium worked the next step was the introduction of streptavidin-coated sepharose beads as a tool to separate binding proteins from residual lysate. Therefore, *E. coli* cell lysate overexpressing Fhit was employed. The experiments were started by incubation of lysate with probe A for 30 min at room temperature, followed by UV irradiation for 30 min. Subsequent CuAAC with N<sub>3</sub>-TAMRA-biotin was followed by incubation with streptavidin beads for 1 h at room temperature in phosphate buffered saline (PBS). The beads were washed with PBS + 0.4% SDS, 6 M urea and PBS and the supernatant was collected after denaturation in SDS loading dye at 95 °C (**Figure 28**).

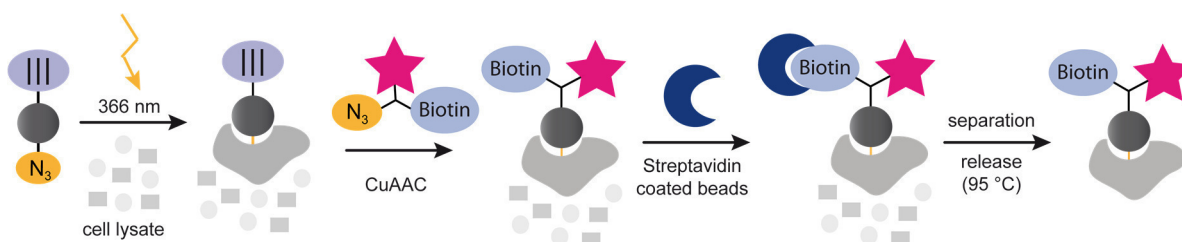


**Figure 28:** Schematic overview on photoreactive ABPP of probe A in HEK 293T cell lysate.

The resulting fluorescent read-out of the SDS-PAGE analysis is depicted in **Figure 29B**. Upon increasing amounts of probe A the Fhit-Ap<sub>3</sub>A-TAMRA-biotin complex was isolated from the beads. This showed that indeed probe A was responsible for labeling of Fhit in the lysate instead of unspecific binding by N<sub>3</sub>-TAMRA-biotin. Additionally, a heat control was performed in which the lysate was heated to 95 °C for 5 min before incubation with probe A. Thus, upon denaturation, Fhit should not be able to bind to probe A anymore. As expected, no band was detected. Interestingly, no further proteins were pulled from the lysate. However, Fhit was overexpressed in the system and might therefore have been the preferable target of probe A.

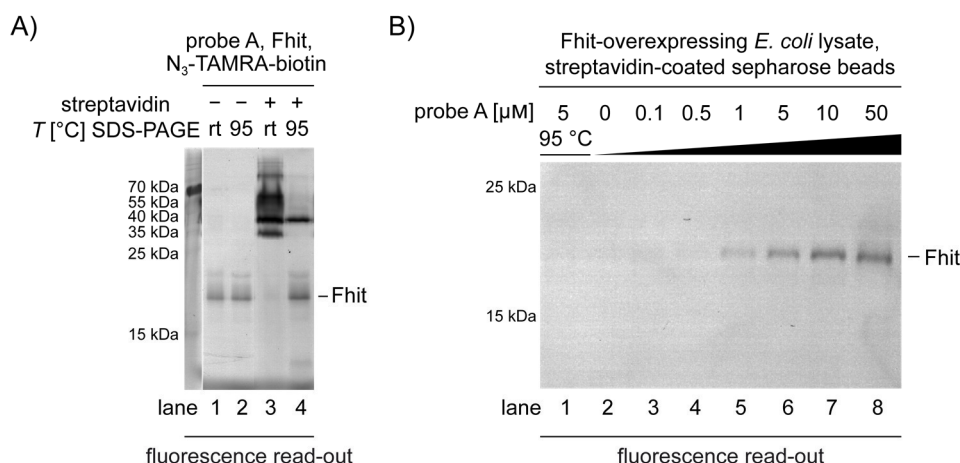


**Figure 29:** Photoaffinity labeling with streptavidin and affinity enrichment with streptavidin beads in *E. coli*. A) Fluorescent read-out of photocrosslinking and subsequent CuAAC with Fhit, probe A and N<sub>3</sub>-TAMRA-biotin, followed by incubation with streptavidin. The temperature indicates the pre-heating of samples before loading the gel. B) Fluorescent read-out of ABPP from Fhit-overexpressing *E. coli* cell lysate using streptavidin-coated sepharose beads.

### 1.5 ABPP in HEK293 T Cell Lysate with Probe A

Although the Fhit overexpressing *E. coli* system was a good proof of principle experiment, interacting partners of Ap<sub>3</sub>A in human cells should be investigated rather than in bacteria. So the next experiments were set up in HEK 293T cell lysates, a Fhit-positive cell line.<sup>180</sup> Similar to ABPP in *E. coli* lysates, probe A was first incubated with lysate, followed by UV irradiation and CuAAC to N<sub>3</sub>-TAMRA-biotin before streptavidin beads were applied. Increasing amounts of probe A were used up to 500 μM in a total volume of 20 μL and 100 μM of probe A in a total volume of 200 μL. From 50 μM on, distinct bands were observed in the fluorescent read-out of the SDS-PAGE analysis (**Figure 30A**). In the larger batch 10 μM seemed already enough to pull proteins from the lysate. The discrimination of these proteins into specific and unspecific binders was the next key point in the investigation of Ap<sub>3</sub>A partners. Hence, control experiments needed to be performed to study the specificity of ABPP and probe A. First of all, proteins unspecifically bound to the streptavidin beads had to be identified. When the input of pure HEK 293T lysate (**Figure 30B**, lane 1) was compared to the outcome after the beads were washed (**Figure 30B**, lane 2) no proteins were visible in the Coomassie-stained gel, implying that the washing procedure worked as requested and removed all unbound proteins. As mentioned before, some unspecific binding might have occurred under the reaction conditions of the CuAAC. Therefore, lysate was incubated in absence of probe A under photoaffinity labeling conditions with subsequent CuAAC with different amounts of N<sub>3</sub>-TAMRA-biotin. Two experiments were conducted in which the beads incubation step was skipped, resulting in distinct bands visible in the fluorescent read-out when 500 μM of N<sub>3</sub>-TAMRA-biotin were applied (lane 4). With only 20 μM (lane 3), however, no proteins were

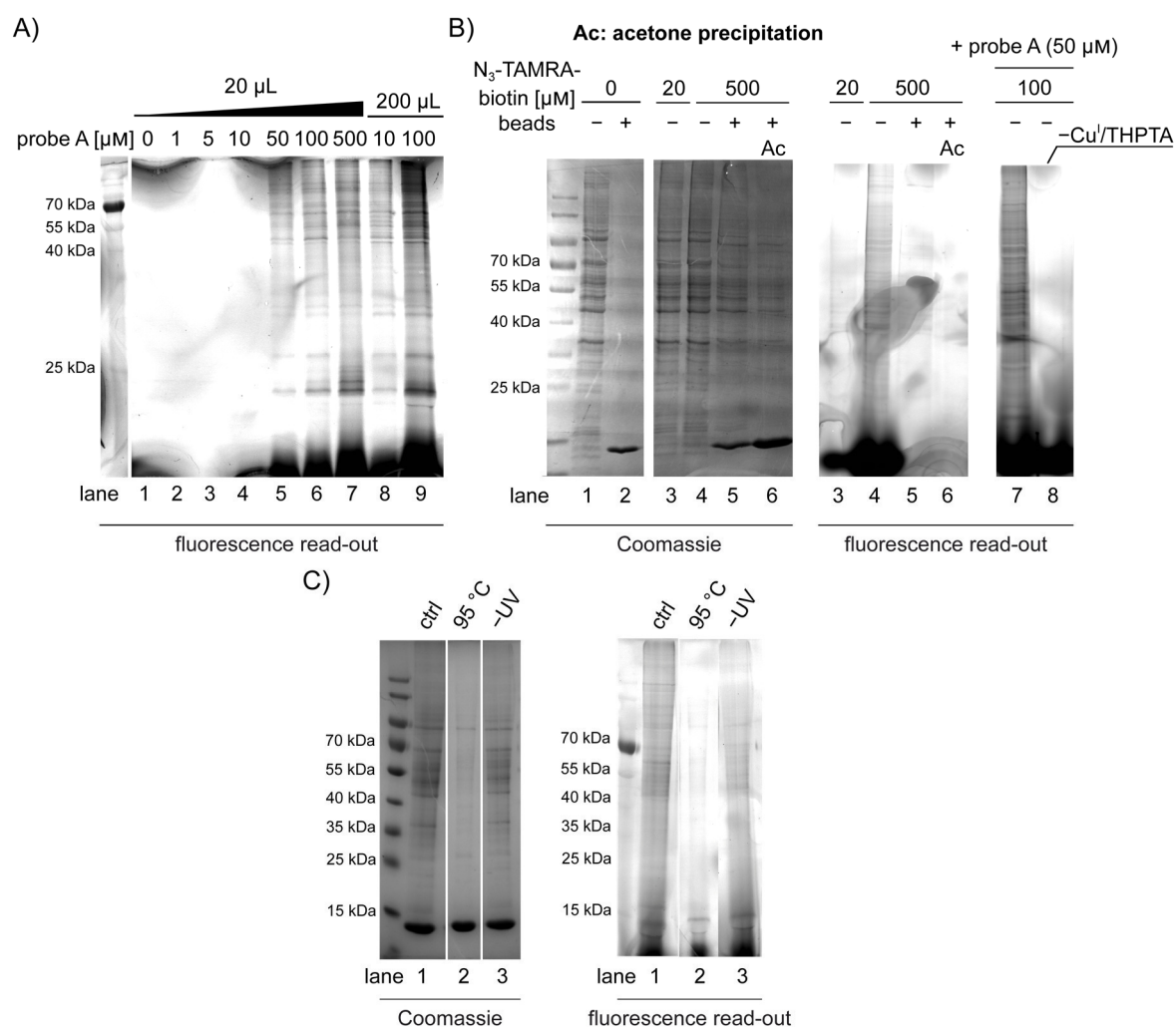


**Figure 30:** ABPP studies in HEK 293T cell lysate. A) Different amounts of probe A were applied in various batch sizes. B) Control reactions without probe A, with and without beads and with and without an additional acetone precipitation in presence of different amounts of N<sub>3</sub>-TAMRA-biotin. Control with probe A and with and without Cu<sup>I</sup>/THPTA. C) Control reactions with probe A with pre-heating and denaturing of lysate and without UV light.

labeled. When the beads incubation plus washing steps were performed, the overall amount of protein was reduced considerably as can be seen in the Coomassie-stained gel (lane 5). The fluorescent read-out showed only small amounts of labeled bands. An additional step of acetone precipitation, added after the CuAAC reaction and before the streptavidin beads, led to a small residual amount of proteins in the Coomassie gel (lane 6). In the fluorescent read-out there were hardly any bands detectable at all. An additional control was made in presence of probe A without Cu<sup>I</sup> and THPTA to investigate if unspecific binding to N<sub>3</sub>-TAMRA-biotin also occurred without the CuAAC reagents. As a result, no bands were detectable in the fluorescent read-out, further indicating that Cu<sup>I</sup> was responsible for unspecific binding (lane 8). The heat control in presence of probe A revealed some protein bands (Figure 30C, lane 2) in the Coomassie gel and equally weak bands in the fluorescent read-out. Additionally, a no UV light control was performed with the HEK 293T lysate and probe A. Protein bands were visible, although, to a lesser extent than in the positive control. In conclusion, a slight amount of unspecific binding should be expected in ABPP experiments and could not be eliminated so far.

## 1.6 Design, Synthesis and Characterization of Competitor Probe B

The discrimination between proteins that were bound in a specific way and the ones bound unspecifically was impossible in the experiment described above. To approach this problem, a competitor molecule should be applied prior to UV irradiation that inhibited binding of probe A. In this way specific proteins would already be blocked by the competitor and could not be approached by probe A. Unspecific proteins, however, would still be able to bind to probe A and could hence be eliminated as interacting proteins of Ap<sub>3</sub>A. Such a competitor should be rather similar to probe A in order to block the respective binding pockets in the same way. This idea was implemented by synthesizing probe B, a non-hydrolyzable Ap<sub>3</sub>A analogue without any modifications at the adenosine moieties and therefore unable to perform ABPP (see **Figure 31**).

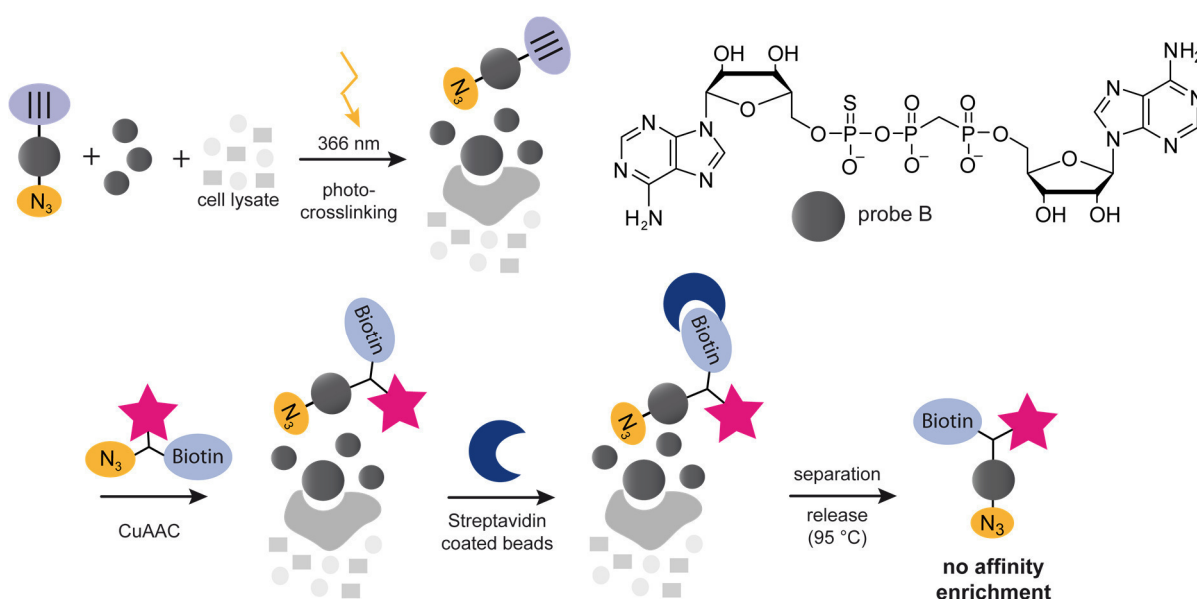
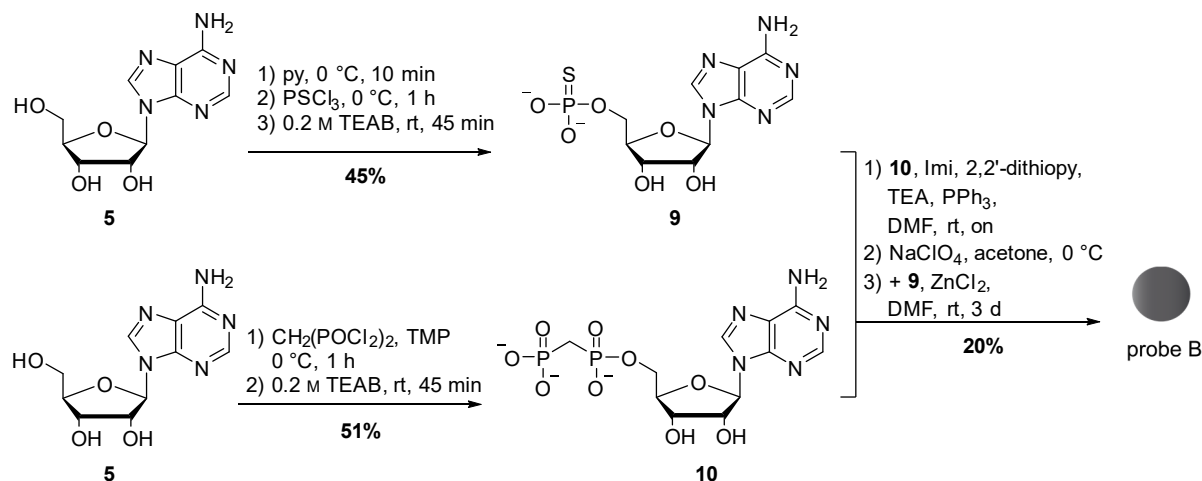


**Figure 31:** Schematic overview on photoreactive ABPP including competitor probe B and its molecular structure.

The synthesis of two building blocks was started from commercially available adenosine (**5**) that was thio-phosphorylated with PSCl<sub>3</sub> in one case and methylene diphosphorylated with CH<sub>2</sub>(POCl<sub>2</sub>)<sub>2</sub> in the other case. Noteworthy is the observation that TMP as solvent was not compatible with phosphorylation via PSCl<sub>3</sub> in case of unmodified adenosine. In these reactions a high amount of methylated thiophosphate was obtained. Therefore, pyridine was used as solvent instead. Three different methods described for Ap<sub>3</sub>A synthesis were then compared with respect to yield and ease for workup. The classical synthesis described by Blackburn *et al.*<sup>239</sup> that comprised thiophosphate **9** activation with diphenyl phosphorylchloride delivered yields around 1% of pure product with separated diastereoisomers. As an alternative, a synthesis originally reported for hydrolyzable Ap<sub>3</sub>As by Mohamady and Taylor was performed.<sup>139</sup> Here, the diphosphate **10** was activated with *N*-methylimidazole in presence of NEt<sub>3</sub> since thiophosphates cannot be successfully activated with imidazoles.<sup>156</sup> The reaction to the dinucleotide, however, was not successful and no product was identified. A third procedure was performed, originally described for the synthesis of dinucleotide tetraphosphates as mRNA

cap analogs containing guanosine instead of adenosine moieties.<sup>156</sup> The synthesis was adapted and the methylene bisphosphonate **10** was activated with imidazole, 2,2'-dithiopyridine, triethylamine and triphenylphosphine. Precipitation with NaClO<sub>4</sub> in acetone led to the activated methylene bisphosphonate imidazolide that was then reacted with **9** under catalysis of ZnCl<sub>2</sub>. The pure diastereoisomers could be obtained in 20% yield. The reaction scheme for the successful ZnCl<sub>2</sub>-mediated synthesis is depicted in **Scheme 14**.



**Scheme 14:** Schematic overview on the synthesis of non-hydrolyzable Ap<sub>3</sub>A probe B starting from commercially available adenosine (**5**).

Probe B was, similarly to probe A, tested for its hydrolytic properties in the analytical HPLC assay when exposed to Fhit and SVPD. As depicted in **Figure 32A** the Ap<sub>3</sub>A analogue is not cleaved by either Fhit or SVPD. Additionally, probe B's properties to inhibit cleavage of FRET-Ap<sub>3</sub>A by Fhit was tested in the FRET assay described for probe A (see **Figure 32B**). Increasing amounts of probe B significantly disturbed cleavage of Ap<sub>3</sub>A, hence, probe B should be a suitable competitor to prohibit binding of probe A to specific proteins.

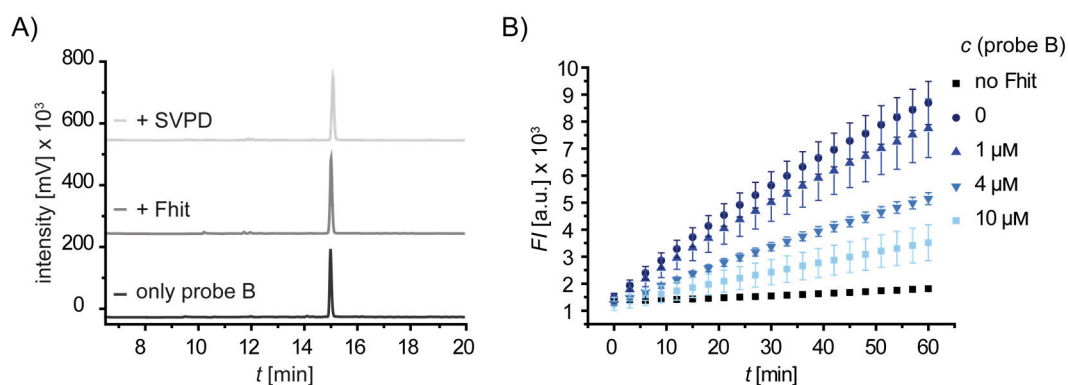


**Figure 32:** Characterization of probe B. A) Result of the analytical HPLC of probe B with and without the enzymes Fhit and SVPD. B) Plot of the fluorescence intensity over time for the hydrolysis of FRET-Ap<sub>3</sub>A by Fhit with increasing amounts of probe B.

## 1.7 ABPP in HEK 293T Lysate with Probe A and Probe B

The competitor was then applied in two experiments in HEK 293T cell lysate in one of which solely probe A and in the other one the fourfold amount of probe B together with probe A were used. Thereby, incubation with probe B was performed prior to the addition of probe A. The resulting Coomassie-stained gel showed a difference of intensities in the entirety of each lane, implying that fewer proteins were labeled when the competitor was present (**Figure 33A**). A closer look into the fluorescent read-out revealed that the overall fluorescence intensity of the two experiments was very similar. However, two distinct lanes were observed that were significantly less intensive in the competition experiment. ABPP was repeated and additionally visualized by Western Blot analysis with Alexa-streptavidin. This read-out showed a slightly different protein pattern than the fluorescent read-out (**Figure 33B**). Nevertheless, again several distinct bands were observed that differed in comparison to the competition experiment.

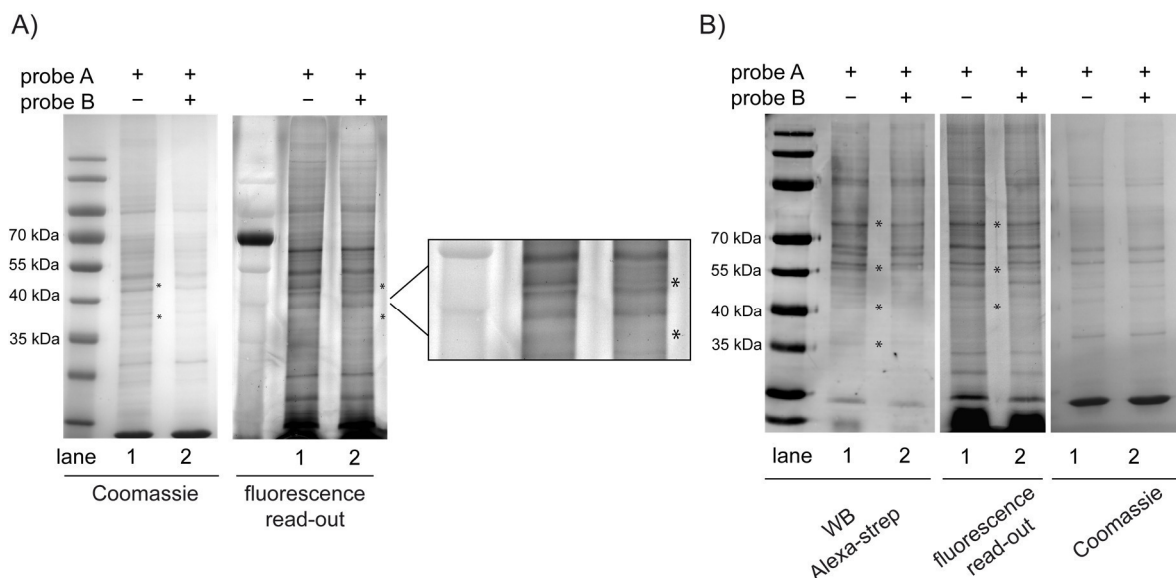


**Figure 33:** Photoreactive ABPP in HEK 293T cell lysate with probe A and B. Comparison between the assay with probe A and a competition experiment in which probe B was first incubated with the lysate before probe A was added. A) Fluorescent read-out and Coomassie-stained gel. B) Experiments with additional WB analysis with Alexa-streptavidin. Stars indicate differences between the lanes.

With these encouraging results preparative experiments were conducted with Fhit-positive HEK 293T cell lysates and some gel bands were transferred to LC-MS/MS analysis. Thereby, the respective bands were chosen from the fluorescence read-out based on the comparison between ABPP with probe A, the competition experiment and a control without any Ap<sub>3</sub>A present. The gel bands were then cut from the Coomassie-stained gel, washed, reduced with DTT, alkylated in the dark with iodoacetamide and digested with trypsin overnight at 37 °C. The pooled supernatant fractions were lyophilized and analyzed with nano-LC-MS/MS. An overview on the procedure is depicted in **Figure 34**. The same experiment was conducted with N<sub>3</sub>-biotin as the CuAAC reagent instead of N<sub>3</sub>-TAMRA-biotin to study if unspecific binding could be reduced by avoiding the dye moiety.

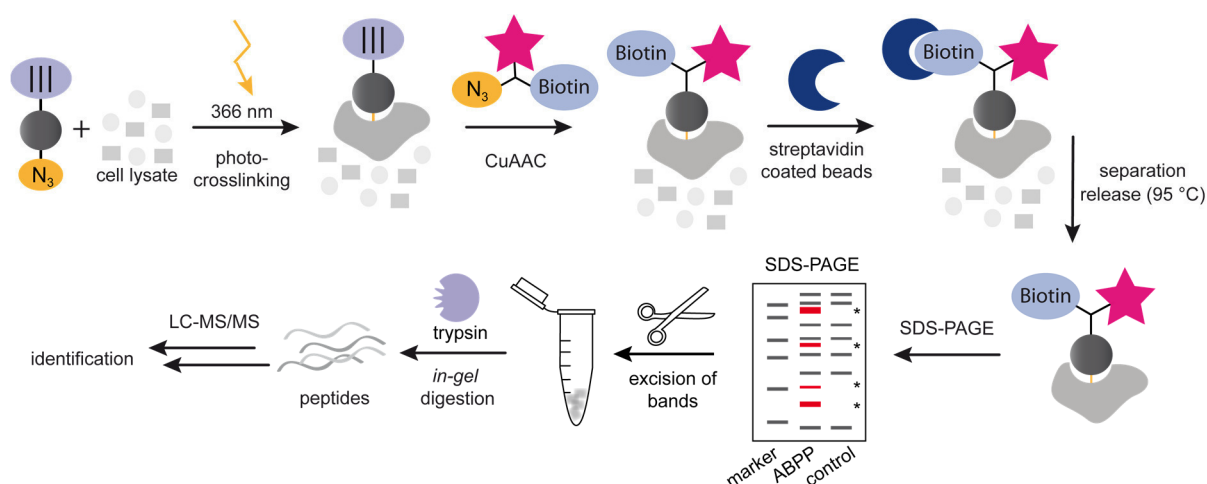


**Figure 34:** Schematic overview on the ABPP procedure utilizing N<sub>3</sub>-TAMRA-biotin including *in-gel* digestion and analysis by LC-MS/MS.

However, the Coomassie-stained gel with N<sub>3</sub>-biotin looked exactly the same as with TAMRA-N<sub>3</sub>-biotin (**Figure 35A**). Since no fluorescent detection was possible in this experiment the whole lane of the respective experiment was cut into four sections and analyzed via LC-MS/MS after tryptic digestion. The analysis of the MS/MS data provided a first hint that phosphatidylethanolamine-binding protein 1 (PEBPI) might be an interacting protein of Ap<sub>3</sub>A since it was discovered with TAMRA-N<sub>3</sub>-biotin and N<sub>3</sub>-biotin in HEK 293T cell lysate and was not detected when the competitor probe B was inside the reaction mixture or when only lysate was bound to the streptavidin-coated beads (see **Table 1** and **Table 2**). PEBPI was identified with scores over 500 and coverages of 35.8% and 52.9%, respectively. These are very high values and a good indicator for accurate MS analysis and data base search. Although this was a quite good first criterion in observing interacting partners of Ap<sub>3</sub>A, the background of the reaction was very high. More precisely, the intensity of the control lane that did not contain any Ap<sub>3</sub>A was as high in the Coomassie-stained gel as all other experiments. Such a high background might be responsible that proteins of lower abundance in the lysates could not be identified by MS/MS. This could also explain why Fhit was not found as a binding protein of Ap<sub>3</sub>A in these experiments. Previous experiments in this thesis already showed that lysate was bound to the beads as long as Cu<sup>I</sup> and THPTA were present. This experiment was repeated and compared to a positive control containing probe A and a negative control without probe A and Cu<sup>I</sup>/ THPTA. The fluorescence read-out clearly showed a high background of unspecifically bound proteins (**Figure 35B**).

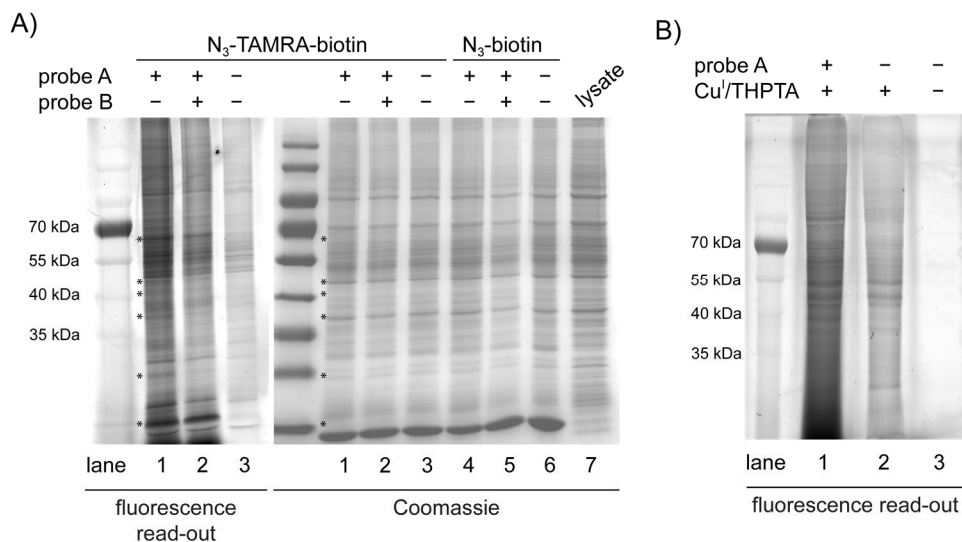


**Figure 35:** ABPP with probe A and B utilizing  $N_3$ -TAMRA-biotin and  $N_3$ -biotin. A) Fluorescent read-out and Coomassie-stained gel of an ABPP experiment from HEK 293T cell lysate with probe A and B. B) Control experiments without probe A and without CuI/THPTA. Stars indicate differences between the lanes.

**Table 1:** Excerpt of proteins identified from ABPP with  $N_3$ -TAMRA-biotin that were absent in the beads and competition controls.

| Protein                                       | Gene  | Score | Coverage [%] | Unique Peptides | MW [kDa] | PSM |
|-----------------------------------------------|-------|-------|--------------|-----------------|----------|-----|
| Phosphatidylethanolamine-binding protein 1    | PEBP1 | 654   | 35.8         | 4               | 21.0     | 12  |
| 78 kDa glucose-regulated protein              | GRP78 | 289   | 6.57         | 2               | 72.3     | 6   |
| Probable ATP-dependent RNA helicase DDX17     | DDX17 | 273   | 5.21         | 1               | 80.2     | 6   |
| Eukaryotic translation initiation factor 5A-1 | EIF5A | 251   | 22.7         | 2               | 16.8     | 3   |

**Table 2:** Excerpt of proteins identified from ABPP with  $N_3$ -biotin that were absent in the beads and competition control.

| Protein                                    | Gene   | Score | Coverage [%] | Unique Peptides | MW [kDa] | PSM |
|--------------------------------------------|--------|-------|--------------|-----------------|----------|-----|
| Tubulin alpha-1B chain                     | TUBA1B | 735   | 25.1         | 7               | 50.1     | 18  |
| Phosphatidylethanolamine-binding protein 1 | PEBP1  | 537   | 52.9         | 5               | 21.0     | 11  |
| Transcription intermediary factor 1-beta   | TRIM28 | 224   | 5.2          | 3               | 88.5     | 6   |
| Stress-induced-phosphoprotein 1            | STIP1  | 209   | 4.8          | 2               | 62.6     | 4   |

The peptide spectrum match (PSM) is an important value when MS/MS data of ABPP experiments are compared since it describes the number of spectra that are obtained for peptides matched to a specific protein.<sup>240</sup> In this way it can be quantitatively compared to other samples. When the PSM number, however, is already very small in the positive sample, it is impossible to decide whether a corresponding peptide in a control sample is really absent or just hidden in the background. The PSM number of PEBP1 was rather small with a value of 11 in the positive controls of both experiments. Hence, the verification as a binding protein had to be postponed until clearer results could be obtained. The overall PSMs were rather small in

the conducted experiments so it was not possible to identify further interaction partners from those ABPP studies.

In conclusion, the conducted experiments with recombinant Fhit, as well as the performed ABPP studies in HEK 293T lysate showed that CuAAC was not compatible with the applied affinity purification strategies. The high background obtained in presence of Cu<sup>I</sup> prohibited a comprehensive and accurate data analysis from the MS results. Hence, at this point the procedure to identify the network of Ap<sub>3</sub>A interaction partners had to be modified by omitting the CuAAC step. Therefore, also a new Ap<sub>3</sub>A analogue needed to be designed and synthesized.

## 1.8 Design, Synthesis and Characterization of ABP Probe C

The crystal structure of Fhit with the non-hydrolyzable Ap<sub>3</sub>A analogue showed free space in the surrounding of position N6 of the outward adenosine.<sup>165</sup> Additionally, the FRET-labeled Ap<sub>3</sub>A analogue was shown to be a substrate of Fhit despite its rather bulky modifications.<sup>222</sup> Hence, an Ap<sub>3</sub>A analogue was chosen that carried the biotin moiety instead of the alkyne handle and should enable ABPP experiments without CuAAC. The molecular structure of probe C is depicted in **Figure 36**.

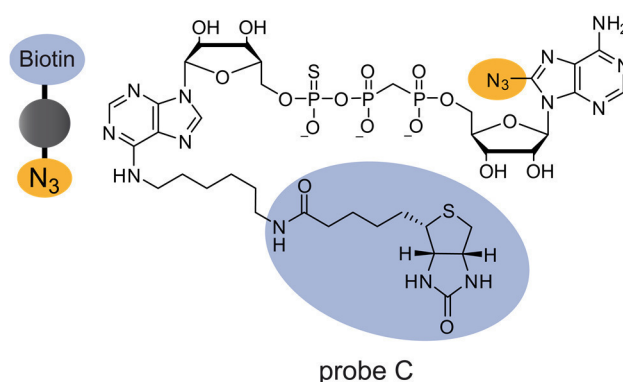
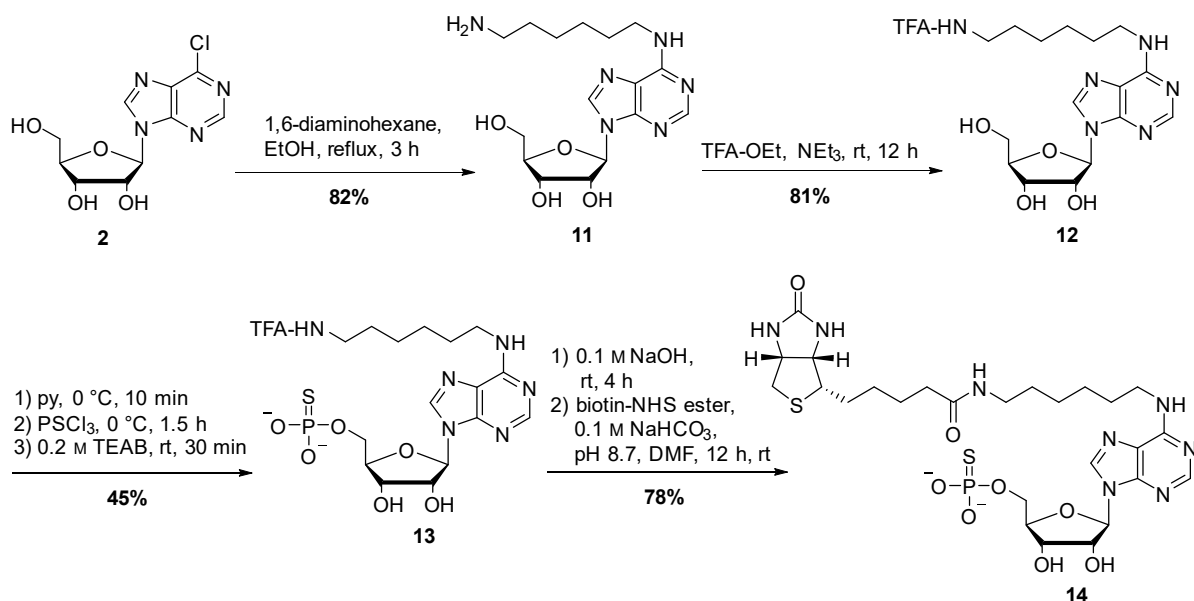


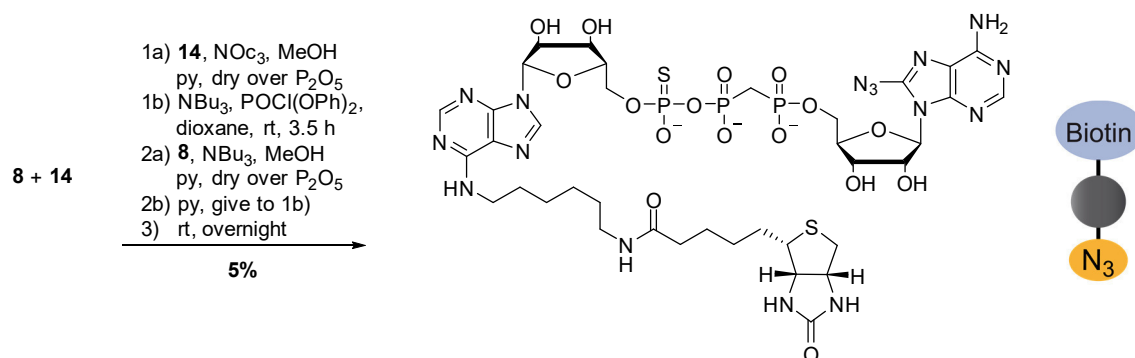
**Figure 36:** Molecular structure of probe C.

The synthesis of the biotinylated non-hydrolyzable Ap<sub>3</sub>A analogue probe C started in analogy to the synthesis of probe A with the modification in position C6 of Cl-inosine (**2**). Instead of the pentynyl linker, commercially available 1,6-diaminohexane in EtOH was reacted with **2** to give the N6-modified adenosine **11**.<sup>241</sup> The free amino group was further protected with TFA-OEt under basic conditions to yield **12**.<sup>242</sup> The protected modified adenosine was thiophosphorylated with PSCl<sub>3</sub> in pyridine as described before to give **13** after purification by anion exchange chromatography and RP-HPLC. In the last step, *in situ* deprotection with 0.1 M NaOH and subsequent reaction with biotin-NHS in NaHCO<sub>3</sub> buffer at pH 8.7 led to building block **14**. Biotin-NHS was prepared as previously described from D-biotin and N-hydroxysuccinimide.<sup>243</sup>



**Scheme 15:** Synthesis of *N*6-biotinylated adenosine 5'-thiemonophosphate (**14**).

Since the photoreactive group should not be changed in probe C, building block **8** was applied again. The coupling to compound **14** was performed similarly to the coupling procedure of probe A following the procedure of Blackburn *et al.*. Activation of thiophosphate **14** with diphenyl phosphoryl chloride was followed by reaction with building block **8** overnight. The desired compound probe C was isolated after several steps of anion exchange and RP-HPLC in 5% yield. Again, also the coupling described by Mohamady and Taylor, as well as the procedure developed for mRNA cap analogues, were performed. Both procedures, however, failed in case of probe C.



**Scheme 16:** Synthesis of probe C.

The successfully synthesized probe C was then tested for its stability properties in presence or absence of Fhit and SVPD at 37 °C for 1 h. Analysis with analytical HPLC revealed the non-hydrolyzability of the synthesized probe C (**Figure 37A**). Furthermore, the FRET assay, already utilized for probe A and competitor B, was applied to probe C. In accordance with the previously obtained results that Fhit also accepts bulky modifications in position *N*6 also probe C inhibited cleavage of FRET- $\text{Ap}_3\text{A}$  when increasing amounts were applied in the assay (**Figure 37B**).

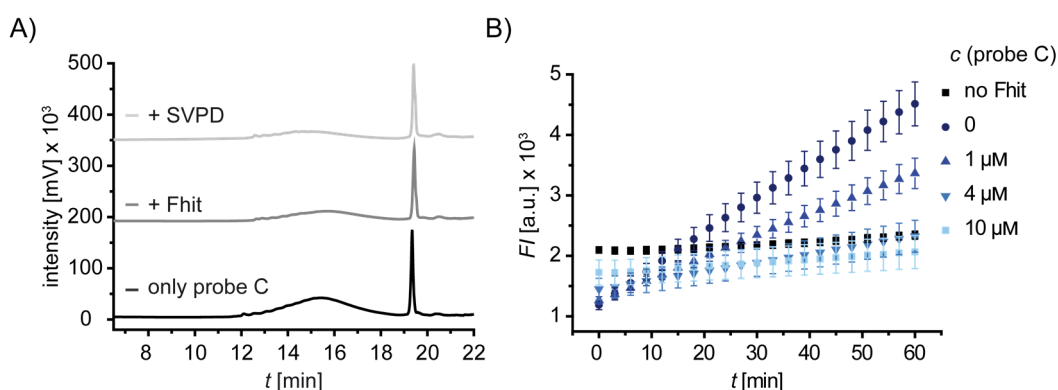


**Figure 37:** Characterization of probe C. A) Results of the analytical HPLC of probe C with and without the enzymes Fhit and SVPD. B) Plot of the fluorescence intensity over time for the hydrolysis of FRET-Ap<sub>3</sub>A by Fhit with increasing amounts of probe C.

## 1.9 Photoaffinity Labeling of Fhit by Probe C

Further analysis of probe C should reveal if it is a better suited reagent regarding the specificity towards Fhit and other potential interaction partners than probe A. In a first experiment the amount of UV irradiation needed for sufficient labeling of Fhit with probe C was determined. The WB analysis with streptavidin-HRP allowed the direct visualization of biotin-labeling of Fhit. As a result, when no UV light was used, Fhit was not labeled (**Figure 38**). After 1 min of irradiation, however, a slight band was already visible on the WB. After approximately 60 min no further labeling occurred. The investigation of probe C also included the ability of labeling unspecific proteins. Therefore, BSA was tested as a protein currently unknown to bind or process Ap<sub>3</sub>A or other dinucleotides. Indeed, BSA was almost not labeled by probe C as visualized in the Coomassie-stained gel and the WB analysis (lane 13, lane 14).

The reaction of Fhit with probe C for 60 min under UV irradiation was also analyzed by mass spectrometry. One peak in the chromatogram resulted in two peaks in the mass spectrum with  $m/z$  values of 17,835.8424 and 16,726.6112, respectively (**Figure 39**). The difference of the two peaks corresponded to 1109.2312  $m/z$ , the mass of probe C in the positive MS mode having lost two nitrogen atoms (calculated 1109.2575). During UV activation of the azide group a nitrene is formed to react with the protein and two nitrogen atoms are leaving as N<sub>2</sub>. The peak of 16.7 kDa matched Fhit with a truncated N-terminal His-tag (calculated 16,726.99), an event that can occur in MS analysis of proteins. The results further indicated that approximately 30% of protein was labeled with probe C.

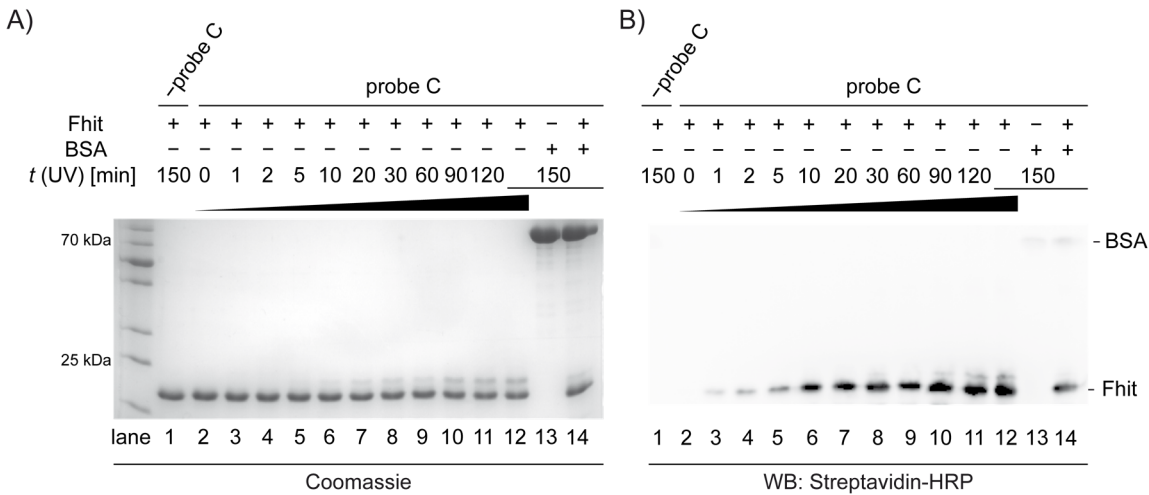


**Figure 38:** Photoaffinity labeling of Fhit by probe C. A) Coomassie gel of a UV irradiation time scale experiment of probe C and Fhit as well as probe C and BSA and probe C, BSA and Fhit. B) Corresponding WB analysis with streptavidin-HRP.

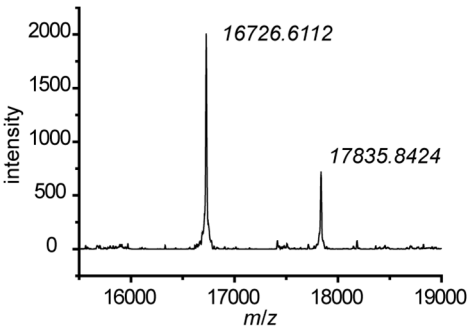


**Figure 39:** HR-ESI-MS spectrum of Fhit labeled by probe C.

Probe C was further investigated towards its usability in ABPP experiments. Therefore, a control was performed in which Fhit was denatured in 2% SDS at 95 °C for 5 min before incubation with probe C. The resulting WB analysis with streptavidin-HRP showed almost no labeling of Fhit (**Figure 40A**). Next, the influence of competitor probe B, already introduced in **chapter III 1.6**, on Fhit labeling was investigated. Upon increasing amounts of probe B titrated to the photoreaction less labeling was observed (**Figure 40B**). When 100  $\mu$ M of probe B were used (10x concentration over probe C) no labeling was observed at all. These encouraging results render probe C a very promising reagent for ABPP experiments in cell lysates.

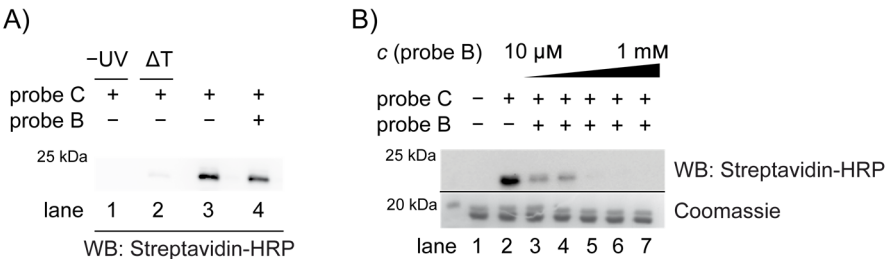


**Figure 40:** Control experiments for photoaffinity labeling of Fhit by probe C. A) WB analysis of the photoreaction between probe C and Fhit (3) without UV light (1), with denatured Fhit (2), with addition of competitor probe B (4). B) top: WB analysis of the photoreaction between probe C and Fhit with increasing amounts of probe B. bottom: corresponding Coomassie-stained gel.

## 1.10 ABPP in HEK 293T Lysate with Probe C and Competitor Probe B

The ABPP experiment that should be applied in HEK 293T cell lysate is schematically depicted in **Figure 41** and should proceed in the following way. In route 1 probe C should be incubated with cell lysate, followed by UV irradiation. Hereupon, a covalent link between probe C and proteins in close proximity should be formed. Incubation with streptavidin-coated sepharose beads was followed by washing, subsequent separation from the residual lysate and elution of the bound proteins. In a second route, competitor probe B should be first incubated with the cell lysate before probe C was added and the UV irradiation was started. The covalent link between probe C and proteins could only occur in case of unspecifically binding proteins since binding pockets of specific binders should be already blocked by probe B. The resulting SDS-PAGE analysis of both routes could be visualized via WB analysis or Coomassie-staining and compared in terms of differences in the protein labeling pattern to identify bands that appear specifically upon treatment with probe C but vanish after addition of competitor probe B. Further analysis of the respective proteins would then be implemented by either *in-gel* tryptic digestion of the protein bands from the Coomassie-stained gel or by direct *on-beads* tryptic digestion prior to protein release from the streptavidin-coated beads. The complex peptide mixtures could then be analyzed by LC-MS/MS and compared to protein databases for identification.

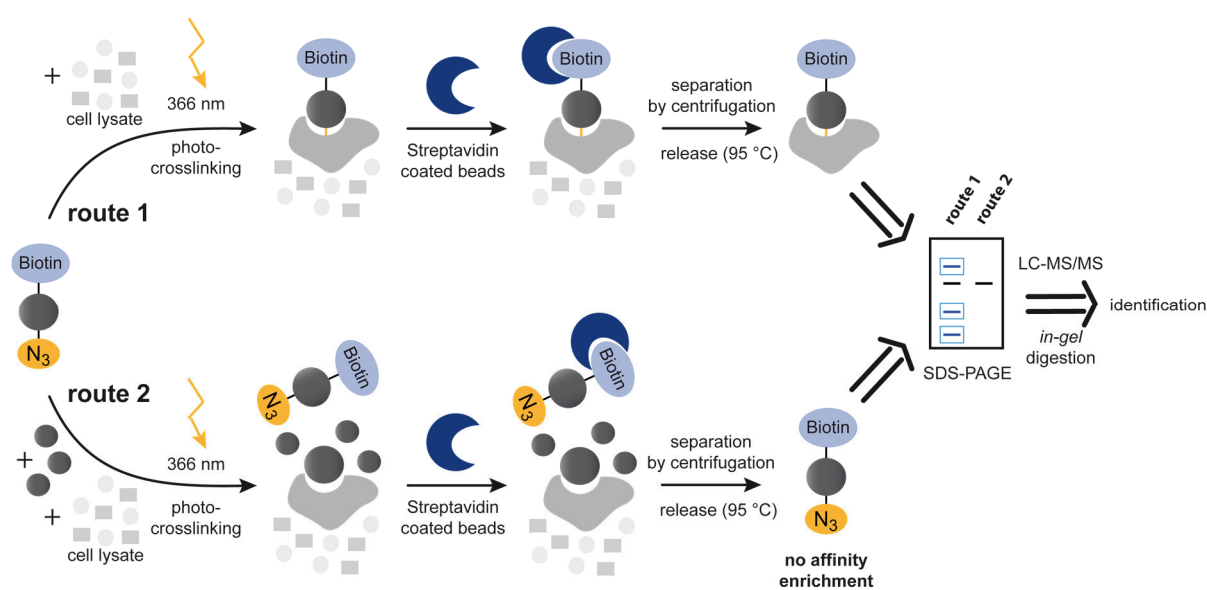


**Figure 41:** Schematic overview on the ABPP procedure conducted with probe C and competitor probe B.

Experimentally, photoreactive ABPP was conducted with a negative control in absence of Ap<sub>3</sub>A and a control in which Fhit was applied additionally to the HEK 293T lysate to observe if probe C is able to label Fhit in the lysate (see **Figure 42A**). The WB analysis via streptavidin-HRP exhibited a strong distinct band in the Fhit control at the size of the protein (lane 3). Further proteins were labeled only slightly. In the negative control without Ap<sub>3</sub>A present no proteins were labeled at all, as expected, since no biotin was present from external sources. The positive

control, however, showed a stronger intensity of protein bands on the WB in comparison to the Fhit control. Samples of a similar Fhit control of a simultaneously conducted experiment were digested with trypsin directly *on-beads* without any prior release from the streptavidin-coated resin. LC-MS/MS measurements of the peptide mixture strongly indicated the band from the WB as Fhit protein. A peptide coverage rate of 73% and a Mascot Score of 364 were sufficient indications for identification as well as the second position in the overall identified proteins list (see excerpt in **Table 3**). Additionally, several other proteins were identified which, however, were not labeled as strongly in the WB. Furthermore, these results demonstrated that Fhit can also be labeled by probe C in the environment of a cell lysate with many different surrounding proteins and metabolites. The identification by LC-MS/MS analysis also provided the important information that the proposed ABPP procedure worked as intended and that *on-beads* tryptic digestion is indeed the right choice to identify further interaction partners of Ap<sub>3</sub>A.

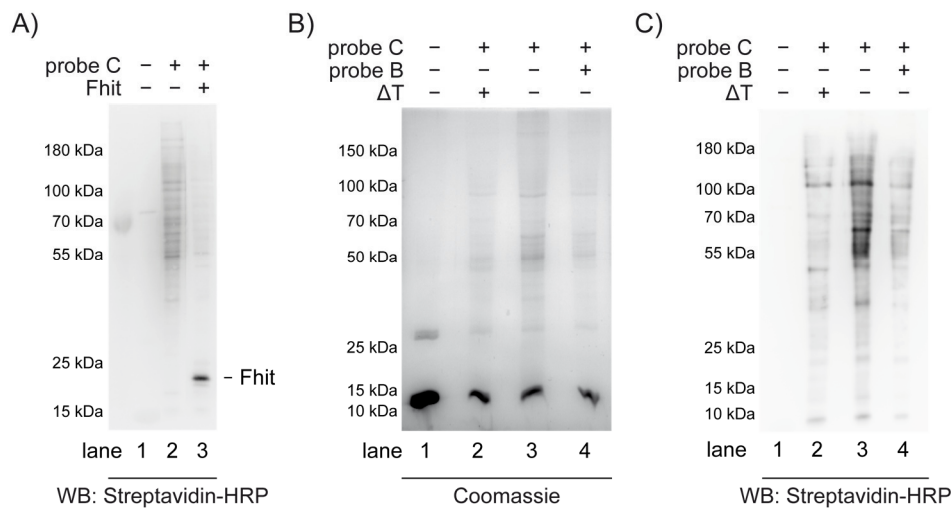


**Figure 42:** ABPP with probe C and B. A) Control experiments without any Ap<sub>3</sub>A (1), positive control with probe C (2) and additional Fhit application (3). B) Coomassie-stained gel of the ABPP experiments without any Ap<sub>3</sub>A (1), heat control (2), with probe C (3), with probe C and tenfold amount of probe B (4.) C) Corresponding WB analysis with streptavidin-HRP.

**Table 3:** Excerpt of the Mascot analysis of LC-MS/MS data obtained from ABPP with Fhit control.

| Protein                           | Gene     | Score | Coverage [%] | Unique Peptides | MW [kDa] | PSM |
|-----------------------------------|----------|-------|--------------|-----------------|----------|-----|
| 60 kDa heat shock protein (mito.) | HSPD1    | 799   | 33.9         | 20              | 61.0     | 28  |
| Bis(5'-adenosyl)-triphosphatase   | FHIT     | 364   | 72.8         | 9               | 16.8     | 15  |
| Heat shock protein HSP 90-beta    | HSP90ABI | 362   | 18.8         | 8               | 83.2     | 14  |
| Tubulin alpha-1A chain            | TUBA1A   | 353   | 24.8         | 8               | 50.1     | 10  |

The next ABPP study was conducted with the same negative control in absence of Ap<sub>3</sub>A, a heat control, in which cell lysate was treated at 95 °C in 2% SDS for 5 min prior to incubation with probe C and the competition experiment in that probe B was applied tenfold over probe C. The Coomassie-stained gel showed a reasonable discrimination in the overall intensity of the protein lanes (**Figure 42B**). In the negative control in absence of Ap<sub>3</sub>A no protein bands were

observed (lane 1). A more intensive lane was exhibited by the heat control (lane 2), however, this was much less intense than ABPP with probe C (lane 3). Application of competitor probe B again resulted in less intense protein signals (lane 4). The same intensity scheme was apparent in the WB analysis (**Figure 42C**) in which no labeling could be observed in the negative lane. However, an even stronger difference between the heat control and the probe C lane was observed in comparison to the Coomassie read-out. Slightly lower protein intensity was detected for the competition experiment. Although the intensities of heat control and competition experiment were rather similar, a distinct labeling pattern was observed, which might be due to the competitor working as intended.

In comparison to the previously obtained results with probe A, the Coomassie-stained gel showed much less proteins in the negative control and also diverse intensities of the proteins were detected from the different ABPP experiments with and without competitor. Therefore, the experiments should be further analyzed by MS. The obtained Coomassie-stained gels were cut into small pieces, destained, digested with trypsin and subjected to LC-MS/MS. The overall amount of identified proteins per lane, however, was much lower when compared to direct *on-beads* digestion. Therefore, SDS-PAGE was exchanged by direct *on beads* tryptic digestion and LC-MS/MS. This method offered the advantage of fewer loss of material since the additional elution plus SDS-PAGE steps were omitted. Furthermore, the ABPP assay was upscaled from 100  $\mu$ L to 1 mL incubation volume including probe C, probe B and beads to obtain further binding proteins that might be of lower abundance and are hidden in small scale experiments.

### 1.11 MS Data Analysis

The preparative ABPP assay with probe C was performed in three biological replicates from individual batches of the respective HEK 293T cells. Thereby, the controls of no Ap<sub>3</sub>A, a heat control and a competition experiment in which probe B was added tenfold compared to probe C were implemented. The peptide mixtures were measured in technical duplicates with nano-LC-MS/MS and further processed with *MaxQuant*. The final analysis was conducted with *Perseus* 1.6.0.2. A Student's t-test was performed to characterize the data statistically and to determine which proteins were significantly enriched in one sample in comparison to another. To be included in the statistical analysis, the respective protein from the ABPP experiments must have been identified in at least four of six replicates. The full lists of all identified proteins in the different experiments are attached in the **Appendix**.

The obtained results showed that 259 proteins were identified from ABPP with probe C that were significantly enriched in the MS data with respect to the beads control in absence of any Ap<sub>3</sub>A. The volcano plot of a Student's t-test represents these results and displays significant hits in pink (**Figure 43A**). The control experiments with competitor B and the heat control were also analyzed with respect to the beads control, since from these results further unspecifically

binding proteins could be excluded from the results. The respective volcano plots are depicted in **Figure 43B** and C and 249 and 284 proteins were enriched, respectively. Additionally, the ABPP experiment with probe C was analyzed in comparison to both, the beads and the heat control. Eight proteins were identified that were significantly enriched opposite both controls (**Figure 43D**). However, no proteins were identified as significantly enriched in the *Perseus* analysis when all three controls were investigated together.

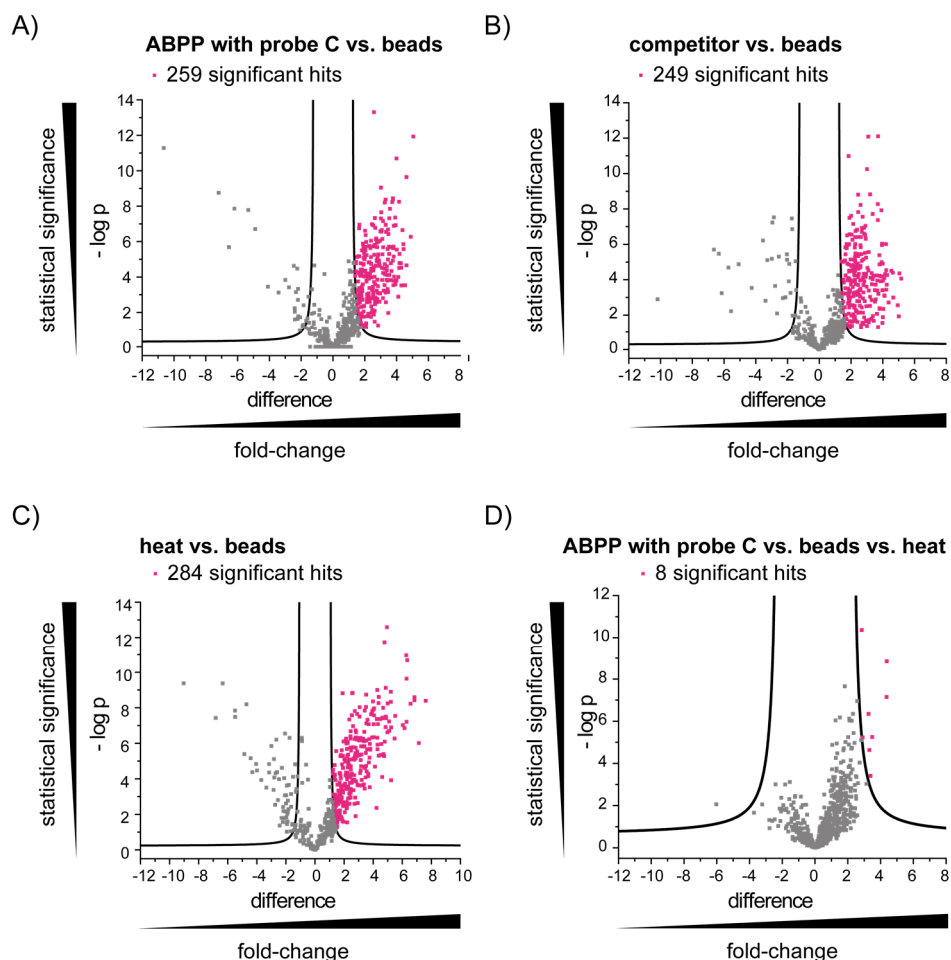


**Figure 43:** Significant enrichment of proteins after ABPP. Volcano plots of the Students t-tests of the respective enrichment over the beads control without any  $\text{Ap}_3\text{A}$ . A) Enrichment of probe C. B) Enrichment of the competition control. C) Enrichment of the heat control. D) Enrichment of ABPP with probe C over heat and beads control.

For this reason, the enriched hits of all experiments were compared individually and several proteins were identified that are absent in both, heat and competition control. The resulting Venn diagrams are depicted in **Figure 44**. Although a high amount of identical proteins was found in all experiments, interestingly, 24 proteins were identified from ABPP with probe C that were solely present in this experiment. Among these is the histidine tRNA synthetase (HisRS). This is especially interesting since aaRS are believed to be responsible for  $\text{Ap}_3\text{A}$  synthesis in the first place. In general, aaRS for 16 of the 20 naturally occurring amino acids were obtained in the list of enriched proteins from ABPP with probe C which is a promising indicator for the applicability of the employed approach.

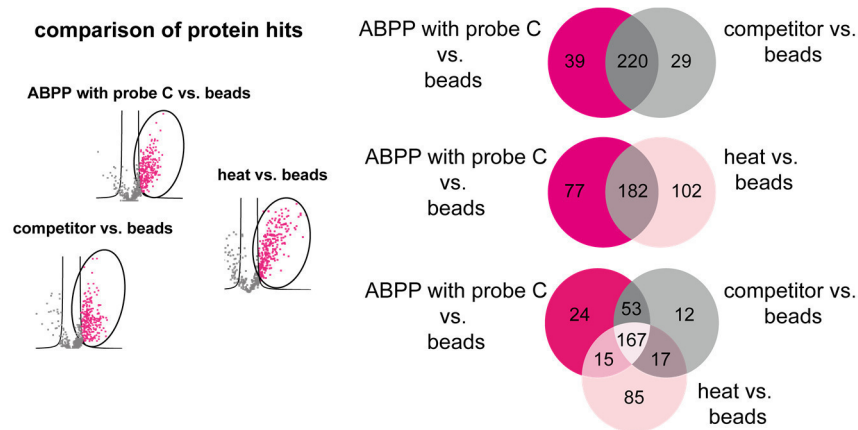


**Figure 44:** Comparison of significantly enriched proteins from ABPP with probe C over beads with enriched proteins from the controls over beads. The resulting Venn diagrams display the amount of overlapping and individually enriched proteins.

The 259 enriched proteins from the ABPP with probe C experiment were then further investigated regarding their molecular function, cellular localization and the physiological processes they are involved in. *Gene Ontology* (GO) terms were used to annotate the respective proteins (**Figure 45**). The majority of proteins were found to be localized in the cytoplasm. Interestingly, multiple potential Ap<sub>3</sub>A interaction partners were annotated to extracellular exosomes. As Ap<sub>3</sub>A is also present in the extracellular space, these proteins might be involved in the outward transportation of intracellularly synthesized Ap<sub>3</sub>A. With respect to their physiological role, no single specific target process was identified within the proteins. This is not surprising since up to now, Ap<sub>3</sub>A was reported to occur in a wide range of biological events.<sup>9</sup> Regarding their molecular function, special attention was focused on nucleotide binding. Due to the similarity of Ap<sub>3</sub>A and ATP, proteins might have been labeled in the lysate that bind Ap<sub>3</sub>A as an ATP surrogate. By contrast, the annotation shows that only 27% of the enriched proteins are known to bind ATP. Furthermore, ADP-binding was annotated to less than 5% of

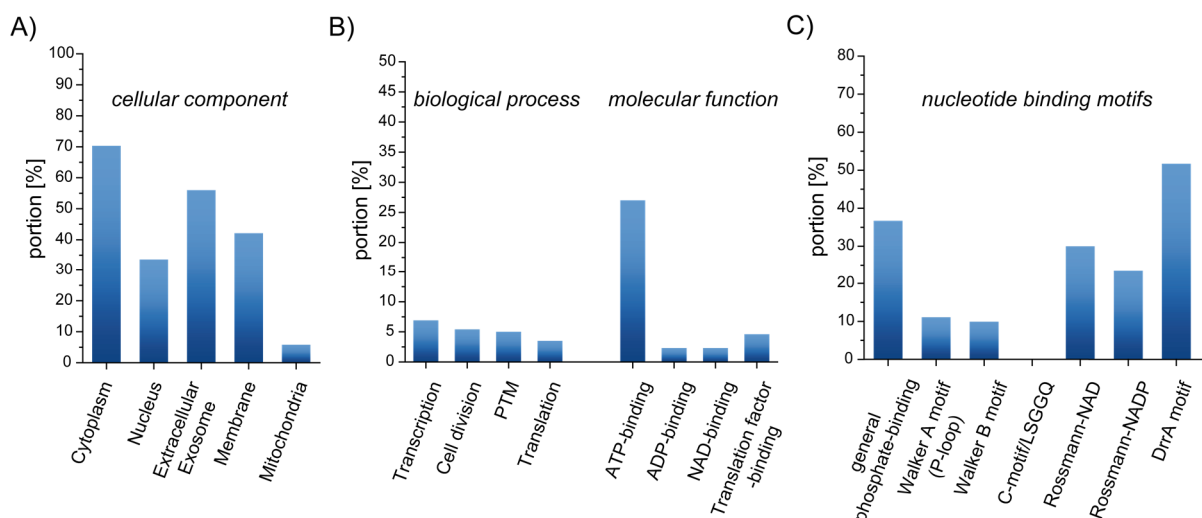


**Figure 45:** Annotation of identified proteins divided into cellular component (A), biological process and molecular function (B). C) Comparison of identified protein sequences to common nucleotide binding motifs.

the proteins. To exclude surrogate-binding to other dinucleotides, NAD-binding was analyzed and also resulted in less than 5%.

To further characterize the potential Ap<sub>3</sub>A interaction partners, the protein sequences were analyzed in detail. Unfortunately, no matching region, domain or sequence was identified. Therefore, the sequences of the identified proteins were compared to known nucleotide binding motifs. Approximately 35% of enriched proteins possess the amino acid motif G-X-X-G-X-G which is reported commonly in phosphate-binding proteins.<sup>244</sup> Specific binding motifs in ATP-binding proteins are the Walker A motif, also known as P-loop motif, the Walker B motif and the C or LSGGQ motif.<sup>245,246</sup> Both of the Walker motifs were found in less than 10% of proteins, the C motif was even absent. To compare dinucleotide binding motifs, the Rossmann folds for NAD- or NADP-binding proteins were compared to the protein sequences.<sup>247,248</sup> Both were present in 20–30% of all enriched proteins. Interestingly, the DrrA motif, reported to be involved in ATP-dependent AMPylation, was observed reccuringly in 50% of identified proteins.<sup>249</sup>

These results suggest that the majority of identified proteins has not yet been associated with nucleotide binding. Indeed, many of these proteins are still uncharacterized. Besides, also the substrate specificity of proteins with known nucleotide binding properties needs to be broadened towards Ap<sub>3</sub>A.

## 1.12 Discussion and Outlook

Summarizing the obtained results, a photoreactive ABPP probe to identify novel interaction partners of Ap<sub>3</sub>A was developed and utilized successfully in competitive ABPP for the first time. Two different Ap<sub>3</sub>A analogues were designed and synthesized to be deployed in one-step and two-step ABPP.

Two-step ABPP is very convenient since only small modifications like alkyne handles need to be attached to the probe that can later be reacted with a sterically-demanding affinity tag. ABPP in HEK 293T lysate with alkyne-modified probe A, however, caused intense background labeling during CuAAC. Hence, a comprehensive discrimination between specifically and non-specifically binding proteins was impossible after MS analysis.

Probe C, equipped with a biotin tag, was successfully applied in one-step competitive ABPP in HEK 293T cell lysates with a suitable competitor probe B. Potential interacting partners of Ap<sub>3</sub>A were detected by quantitative label-free MS analysis. The MS data was evaluated with a proteomics-based statistical software to obtain significant enrichment of proteins in one sample over another. An advantage of the utilized ABPP assay is the detection of a broad range of proteins opposed to a single class of enzymes that is revealed in activity-based studies.<sup>250</sup>

The results revealed multiple proteins that had no overlap with previously identified interaction partners of other Ap<sub>n</sub>A family members, implying a distinct biological role for Ap<sub>3</sub>A. Several aaRS were obtained, which are known to be involved in the synthesis of Ap<sub>3</sub>A. These findings demonstrated the potential of the applied ABPP approach. The identified proteins were further analyzed regarding their cellular localization. Although mostly cytosolic, multiple proteins were located in extracellular exosomes. With respect to the extracellular signaling pathways Ap<sub>3</sub>A is involved in, these results provide valuable information about the proteins that participate in these processes.

Detailed analyses of the protein sequences revealed that only one quarter of identified proteins were known ATP-binders, suggesting that Ap<sub>3</sub>A was not bound as an ATP surrogate. Thus, in contrast to the similarity between these compounds, this is further evidence for their various physiological purposes. The majority of interaction partners has not yet been associated with nucleotide binding before, indeed, many proteins are even uncharacterized with respect to their functionalities. Hence, an additional characteristic for Ap<sub>3</sub>A binding or processing is highly probable.

Until now, no common characteristic amino acid sequence has been identified, neither for Ap<sub>n</sub>A in general nor for Ap<sub>3</sub>A in particular. Therefore, the sequences of the identified proteins were analyzed with respect to mutual motifs and domains. However, no common motif between the sequences was discovered so far. X-ray crystallography can be a useful tool to investigate the binding pockets of the respective proteins bound to Ap<sub>3</sub>A and might lead to the identification of a mutual structural motif for Ap<sub>3</sub>A recognition, binding or processing.

As a next step, the identified proteins should be validated individually by biophysical and mechanistic studies to reveal their role in Ap<sub>3</sub>A processing. Thereby, the effect of Ap<sub>3</sub>A in the respective signaling pathways, as well as its effect on enzymatic activities will be of particular interest. Furthermore, knock-down experiments with siRNA and the influence of stress conditions might reveal information on the physiological role of Ap<sub>3</sub>A.

Overall, various ABPP probes with differing photoreactive groups, spacers and affinity tags next to experiments with hydrolyzable variants in Fhit-negative cell lines and cell-permeable probes will contribute to a complete picture of the Ap<sub>3</sub>A interactome.

## 2 Small Molecule Inhibitors of the Tumor Suppressor Fhit

The second project of this thesis is about the identification and development of small molecule inhibitors of the enzymatic function of the tumor suppressor Fhit. Mechanisms of protein regulation and Fhit's exact role in tumor suppression are still widely unclear. Hence, to approach this lack of knowledge small molecule inhibitors may be an important tool to further investigate Fhit *in vitro* and *in vivo*. Moreover, a non-hydrolyzable Ap<sub>3</sub>A analogue has already been shown to not only inhibit Fhit's enzymatic activity but to also lead towards Fhit-dependent and caspase-directed apoptosis.<sup>223</sup> Thus, new small molecule inhibitors of Fhit might also be useful in the development of new anti-cancer drugs. To date only a few small molecules have been presented as inhibitors of Fhit's hydrolytic activity to cleave Ap<sub>3</sub>A into AMP and ADP. Among these, Suramin and transition metal ions suffer from unspecificity because they affect several different classes of enzymes or have an even wider range of functions inside human cells.<sup>217,219</sup> Hence, there is a high demand for highly potent inhibitors of Fhit's enzymatic activity. This project was started in cooperation with Stephan M. Hacker in 2014 and followed up together with Philipp Schmid within his Bachelor thesis in 2016.<sup>251</sup>

### 2.1 High-Throughput Screening

In a high-throughput fashion small molecule libraries were screened to identify compounds that reduced the hydrolytic activity of Fhit. A doubly labeled hydrolyzable Ap<sub>3</sub>A analogue was synthesized as previously described by Hacker *et al.*<sup>222</sup> that is able to undergo FRET (see **Figure 46**). The same molecule was already introduced in **chapter III 1.3** of this thesis. Upon cleavage by Fhit the Sulfo-Cy3 and Sulfo-Cy5 dye moieties are decoupled and spatially too far away from each other to undergo FRET. Hence, the donor Sulfo-Cy3 cannot transfer its energy to Sulfo-Cy5 anymore. In a fluorescence intensity read-out, the donor channel of Sulfo-Cy3 can be

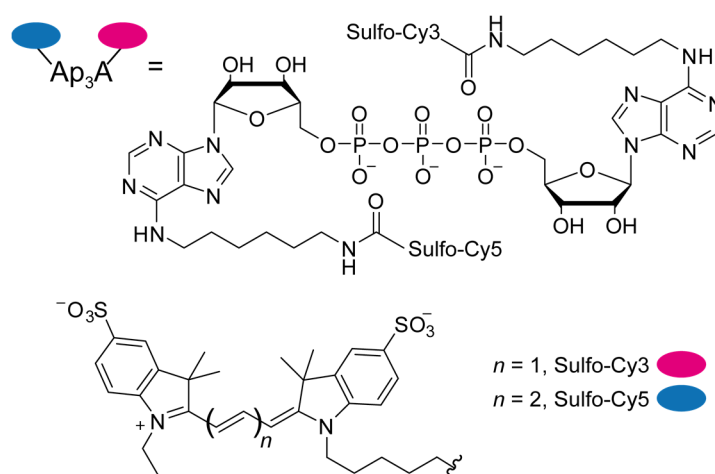


**Figure 46:** FRET-Ap<sub>3</sub>A, carrying the FRET pair Sulfo-Cy3/Sulfo-Cy5, depicted in pink and blue.

measured at 535 nm excitation and 590 nm emission. The fluorescence intensity increases drastically when  $\text{Ap}_3\text{A}$  is cleaved as the energy is emitted instead of being transferred to the acceptor. In this way the cleavage can be followed readily by fluorescence measurements and the influence of small molecule inhibitors on this reaction can be investigated.

15,136 compounds were tested from random small molecule libraries in a high-throughput approach. In 384-well plates the respective small molecules (10  $\mu\text{M}$ ) were preincubated with Fhit for 30 min at 25 °C. FRET- $\text{Ap}_3\text{A}$  was added and the first time point was measured immediately. After 30 min the fluorescence intensity was measured again and the data of these two time points were compared and calculated relative to a DMSO control in absence of any small molecule. The resulting data was analyzed regarding Fhit activity and in total 45 compounds were found that inhibited Fhit's enzymatic activity by over 90% (**Figure 47** and **Appendix**)

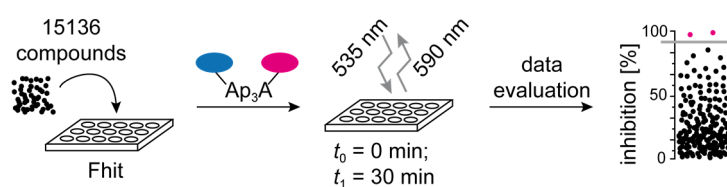


**Figure 47:** Schematic overview on the high-throughput screening.

Among these hits one recurring structural motive was found that showed inhibition within several different substitution patterns. The structure could be determined as dimethyl-hexahydro-quinolinone. This family was divided into two main subgroups with a methyl-pyrazolo group bridging positions  $\text{R}^1$  and  $\text{R}^2$  (Cluster I) and an indeno residue substituted at  $\text{R}^1$  and  $\text{R}^2$  (Cluster II, **Figure 48A**). The best hit of the screening, compound **18a** (**Figure 48B**), was the only compound in the high-throughput screening containing the methyl-pyrazolo group of Cluster I. It inhibited Fhit down to a residual activity of approximately 2%. Toluyl- (**15b**), 4-ethylphenyl- (**16b**) and 2-thiophenyl- (**23b**) substituted derivatives at  $\text{R}^3$  were also found among the best 45 hits carrying the indeno residue at  $\text{R}^1$  and  $\text{R}^2$  and inhibited Fhit activity by more than 95% (**Figure 48B**). An analysis of all compounds belonging to this family in the small molecule libraries gave the result that bulky groups at  $\text{R}^3$  significantly compromise inhibitory activity. Hence, this part of the molecule might be tightly bound to the protein. Compounds that were substituted at the secondary amine also showed decreased inhibitory potential. To improve the properties of inhibitor **18a** derivatives were synthesized that had the two main subgroup motives at  $\text{R}^1$  and  $\text{R}^2$  and different substituents at  $\text{R}^3$ .

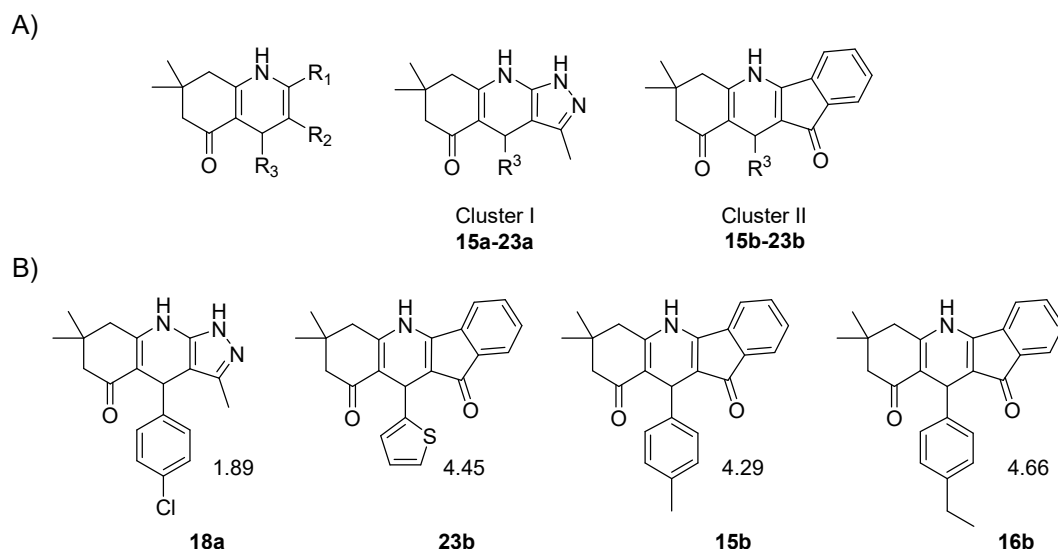


**Figure 48:** Structure of main motives. A) Main motive found reccuringly in the best 45 hits of the high-throughput screening, divided into cluster I and cluster II. B) Compounds that were already found among the best 45 hits of the screening that fit the main motive. Numbers indicate the residual Fhit activity in %.

## 2.2 Synthesis of Potential Inhibitor Derivatives

The synthesis of all 18 derivatives was carried out together with Philipp Schmid within his Bachelor thesis in a one-pot procedure with microwave-assisted heating. Cluster I was synthesized, starting from commercially available dimedone, 3-amino-5-methyl-pyrazole and the respective aldehydes.<sup>252,253</sup> The three reagents were mixed in a 1:1:1 ratio, 1.2 eq.  $\text{NEt}_3$  were added and the mixture was heated in aqueous medium to 170 °C in a microwave for 15 min. Addition of EtOH/ $\text{H}_2\text{O}$  (1:1) led to precipitation of the crude product that was filtered, washed and dried *in vacuo*. The synthesis of compounds **15a-23a** was previously reported in the literature, except for compound **16a** that was synthesized analogously. Cluster II derivatives were made from 1,3-indanedione, 5,5-dimethyl-3-amino-cyclohex-2-enone and the respective aldehydes. To a 1:1:1 ratio of these reagents, *p*-TsOH was added and the reaction was performed in aqueous medium at 150 °C for 10 min. After quenching with NaOH (10%) the compounds were purified by silica gel column chromatography and RP medium pressure column chromatography (MPLC). The synthesis of compounds **18b** and **19b** has previously been described in the literature.<sup>254,255</sup> In total 18 compounds have been synthesized, eight thereof for the first time. The respective yields are listed in **Figure 49**.

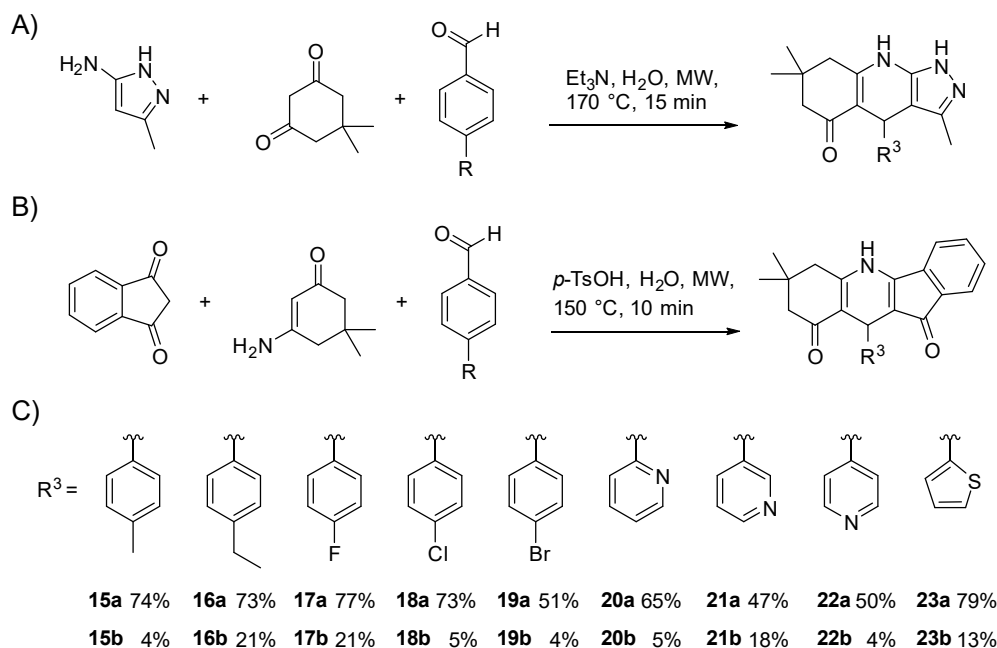


**Figure 49:** Synthesis of derivatives. A) Synthesis scheme of cluster I derivatives. B) Synthesis scheme of cluster II derivatives. C) Synthetic yields obtained from the respective derivatives of cluster I and cluster II.

## 2.3 *In Vitro* FRET Dose-Response Assay

To confirm the results from the high-throughput screening and to further investigate the newly synthesized inhibitors an *in vitro* dose-response assay was employed. The experiment was set up similarly to the screening procedure with fluorescence measurements of the donor Sulfo-Cy3 at 590 nm emission, when excitation at 535 nm was used. Time points, however, were taken every 3 min over 60 min to get a closer look into the kinetics of FRET-Ap<sub>3</sub>A cleavage and the influence of the inhibitor (**Figure 50A**). Furthermore, a linear time response of the cleavage of the substrate would be preferable for later calculation of inhibition. Therefore, the Fhit concentration for hydrolysis had to be determined accordingly. Recombinant Fhit was applied from 0 to 8 nM and already 1 nM concentration gave a satisfying result (**Figure 50B**).

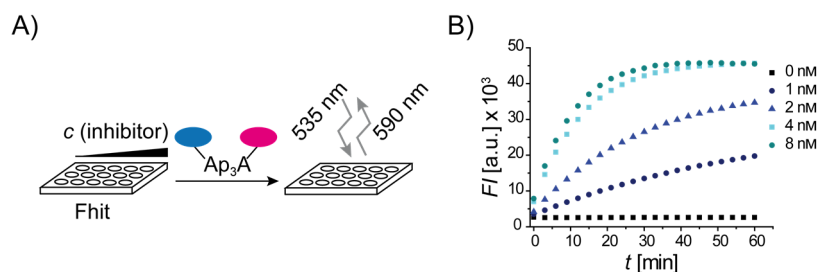


**Figure 50:** *In vitro* FRET assay. A) Schematic overview of the *in vitro* FRET assay. B) Fluorescence intensity against time of different concentrations of Fhit to determine the ideal concentration of the protein for a linear time response of the cleavage of FRET-Ap<sub>3</sub>A.

The *in vitro* FRET assay was conducted in a dose-response manner with increasing amounts of the respective compound from 5.08 nM up to 100  $\mu$ M when 0.5 nM FRET- $\text{Ap}_3\text{A}$  and 1 nM Fhit were applied. As a first test, the best hit of the screening, compound **18a**, was tested and the resulting data of the fluorescence intensity over time is depicted in **Figure 51A**. To obtain the  $\text{IC}_{50}$  value the results were plotted as % inhibition against the logarithmic compound concentration and determined as  $199 \pm 10$  nM for compound **18a** (**Figure 51B**).

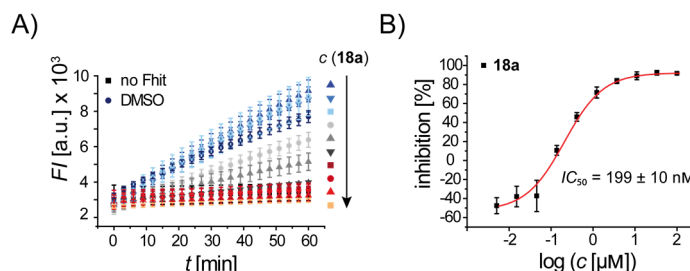


**Figure 51:** Dose-response assay. A) Fluorescence intensity of a dose-response assay of compound **18a** applied in different concentrations. B) Corresponding dose-response curve to determine the  $\text{IC}_{50}$  value. Inhibition in % is plotted against the logarithmic concentration.

The other compounds were then investigated likewise for their inhibitory potential towards Fhit's enzymatic activity (**Figure 52**). Among the potential inhibitors four compounds were found that inhibited Fhit even more potently than lead compound **18a**. The respective structures derived from the indeno-derivatized family and were substituted with ethyl (**16b**), F (**17b**), Cl (**18b**) and Br (**19b**). Interestingly, all derivatives of this cluster II are better inhibitors than the respective derivatives of cluster I when compounds with the same  $\text{R}^3$  group are compared.

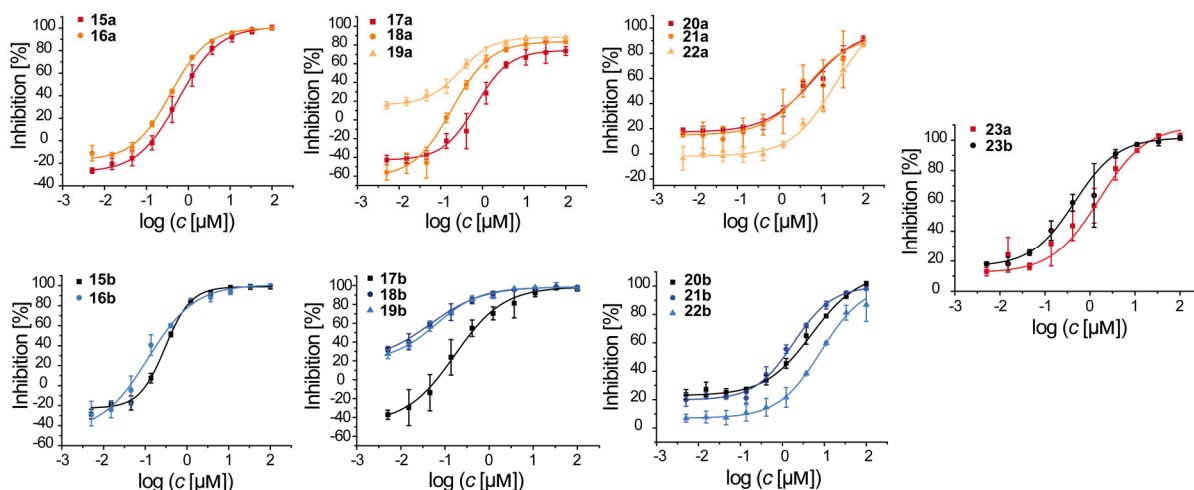


**Figure 52:** Dose-response curves of all synthesized compounds. Inhibition in % is plotted against the logarithmic concentration of the respective inhibitor. Yellow to red curves are compounds of cluster I, blue to black graphs represent compounds of cluster II. The red and black curves correspond to the thiophenyl-substituted compounds.

The overall best inhibitory properties were found for compound **19b** with the Br residue, closely followed by **18b** with a Cl residue. **19b** inhibited Fhit with an  $IC_{50}$  value of  $49 \pm 7$  nm, an  $IC_{50}$  value of  $51 \pm 32$  was calculated for compound **18b**. Among the synthesized derivatives the halogenated (**17–19**) and alkylated (**15, 16**) structural motives seem to have a higher inhibitory potency than the pyridinyl- (**20–22**) and thiophenyl- (**23**) substituted derivatives. This might be due to their altered electronic characteristics in the aromatic systems. Interestingly, some of the compounds, e.g. compounds **17a** or **16b**, seem to have a positive effect on Ap<sub>3</sub>A cleavage when applied in very low concentrations and show their inhibitory potential only when higher amounts are used in the assay. A more complicated inhibitory mechanism might be an explanation for such a behavior. The summarized  $IC_{50}$  values for all compounds are shown in Table 4.

**Table 4:**  $IC_{50}$  values of all synthesized compounds.

| Compounds<br>Cluster I | $IC_{50}$ [nM]     | Compounds<br>Cluster II | $IC_{50}$ [nM]  |
|------------------------|--------------------|-------------------------|-----------------|
| <b>15a</b>             | $589 \pm 160$      | <b>15b</b>              | $275 \pm 1$     |
| <b>16a</b>             | $388 \pm 26$       | <b>16b</b>              | $125 \pm 44$    |
| <b>17a</b>             | $762 \pm 105$      | <b>17b</b>              | $154 \pm 55$    |
| <b>18a</b>             | $199 \pm 10$       | <b>18b</b>              | $51 \pm 32$     |
| <b>19a</b>             | $379 \pm 54$       | <b>19b</b>              | $49 \pm 7$      |
| <b>20a</b>             | $6,682 \pm 1,948$  | <b>20b</b>              | $4,245 \pm 459$ |
| <b>21a</b>             | $2,225 \pm 721$    | <b>21b</b>              | $1,654 \pm 241$ |
| <b>22a</b>             | $16,840 \pm 3,160$ | <b>22b</b>              | $5,204 \pm 261$ |
| <b>23a</b>             | $925 \pm 198$      | <b>23b</b>              | $499 \pm 187$   |

It was important to exclude that the compounds themselves had an influence on the FRET assay by either interacting with the dye moieties of FRET-Ap<sub>3</sub>A or by having fluorescent characteristics that interfered with the read-out. Therefore, a negative control was conducted in which no Fhit was applied and only the influence of the respective compounds on FRET-Ap<sub>3</sub>A and the read-out over 1 h was studied. As displayed in Figure 53 none of the investigated compounds showed an effect on the experiment without Fhit.

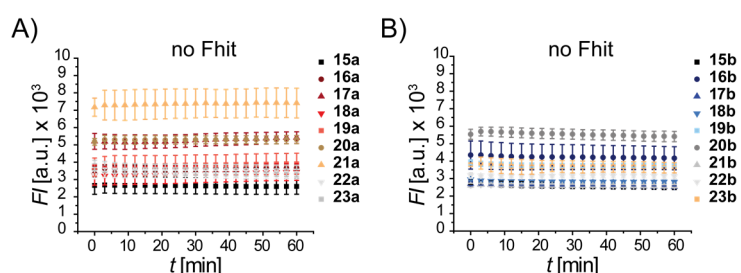


**Figure 53:** Negative control without Fhit to study the influence of the respective compounds on FRET-Ap<sub>3</sub>A and the fluorescent read-out. A) Cluster I derivatives. B) Cluster II derivatives.

## 2.4 Determination of Cellular Toxicity by MTT Assay

HEK 293T and H1299 are two commonly studied cell lines in Fhit and Ap<sub>3</sub>A research.<sup>180,182,223</sup> While HEK 293T are Fhit-positive,<sup>25</sup> the lung cancer cells H1299 have lost the ability to express the tumor suppressor Fhit.<sup>186</sup> Therefore, these human cells are of great interest when Fhit's tumor suppressor functionality is investigated in more detail. If the synthesized derivatives shall be utilized in Fhit studies, it is necessary to know their cytotoxic potential in these cells. Hence, a colorimetric assay was conducted to study the viability of human cells when increasing amounts of the compounds were applied. The assay was based on the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which is reduced to water insoluble formazan by NAD(P)H-dependent oxidoreductases.<sup>256</sup> Thereby, the metabolic activity of cells can be determined. Generally, HEK 293T cells were more sensitive towards the Fhit inhibitors than H1299. 10  $\mu$ M, already, showed a cell-death inducing effect in HEK 293T cells, while the lung cancer cells were not even strongly affected when up to 100  $\mu$ M of inhibitor were used. When compounds from cluster I and cluster II were compared, cluster II compounds showed a slightly higher cytotoxicity than cluster I compounds. This difference was, however, more pronounced in HEK 293T than in H1299. Compounds containing alkylated

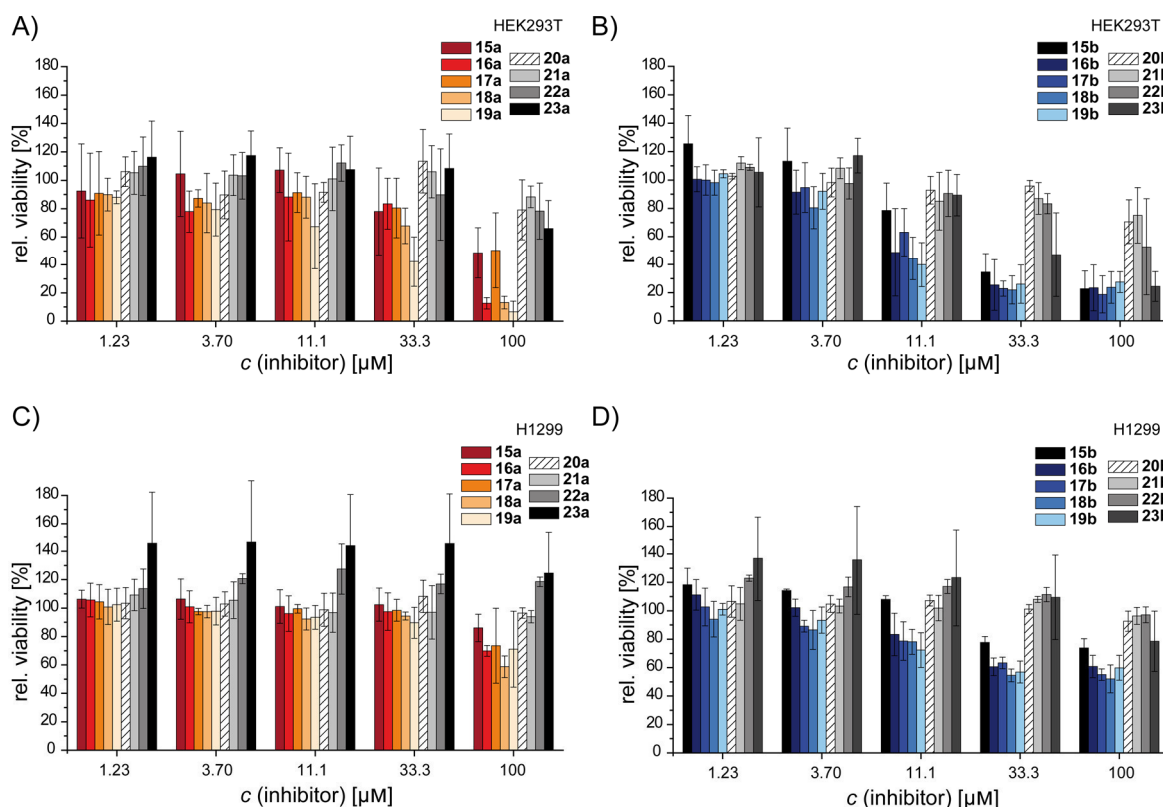


**Figure 54:** Results of MTT assays. The bar chart depicts the relative cell viability in % against the concentration of the respective inhibitors in  $\mu$ M from the employed MTT assay. A) Cluster I compounds in HEK 293T cells. B) Cluster II compounds in HEK 293T cells. C) Cluster I compounds in H1299 cells. D) Cluster II compounds in H1299 cells.

(15, 16) and halogenated (17–19) residues had a stronger influence on the viability of the cells than the pyridinylated (20–22) and thiophenylated (23) compounds. The thiophenylated inhibitors only had a little effect, while the pyridinylated inhibitors showed no cytotoxic potential at all. The relative viability against the concentration of all synthesized inhibitors is depicted in Figure 54.

## 2.5 Further Characterization of the Identified Inhibitors

The next step was to investigate whether cleavage of natural Ap<sub>3</sub>A by Fhit was also inhibited by the compounds to exclude the possibility that an interaction between the Sulfo-Cy3/5 dyes and the inhibitors was responsible for the inhibition. Therefore, an analytical HPLC assay was set up in which the cleavage of Ap<sub>3</sub>A into AMP and ADP was followed. Upon addition of increasing amounts of compound **19b** cleavage of Ap<sub>3</sub>A was clearly inhibited as is depicted in Figure 55A. Thereby a ratio between 1:100 and 1:1000 of Fhit to inhibitor was necessary to interrupt the cleavage of Ap<sub>3</sub>A.

To obtain further information about the type of inhibition of compound **19b** an experiment was conducted in which increasing amounts of FRET-Ap<sub>3</sub>A were incubated with a steady amount of **19b** and cleavage of FRET-Ap<sub>3</sub>A was followed by fluorescence measurements. The obtained results showed that inhibition by compound **19b** was reversed when increasing concentrations of substrate were added to the reaction (Figure 55B). For each FRET-Ap<sub>3</sub>A concentration also a DMSO control without **19b** was conducted.

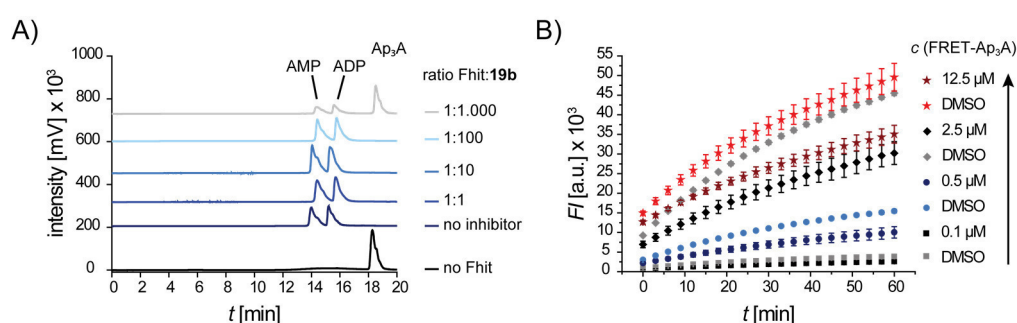


**Figure 55:** Natural Ap<sub>3</sub>A and reversibility of the assay. A) Result of an analytical HPLC assay analyzing the cleavage of natural Ap<sub>3</sub>A by Fhit when increasing amounts of compound **19b** were applied. B) Increasing concentrations of FRET-Ap<sub>3</sub>A were incubated with inhibitor **19b** to study the reversibility of the reaction. Each experiment was also conducted with the respective DMSO control at the same FRET-Ap<sub>3</sub>A concentration.

Fhit is part of the Hit protein family which members all share a conserved triple histidine motive in their active center. In humans, there are seven members of the Hit protein family, including Fhit.<sup>74,257</sup> Since the high-throughput screening was not designed to discriminate between the different Hit members there is the possibility that the inhibitors also effect the enzymatic reactions of the other members. Therefore, an analytical HPLC assay was conducted in which HintI was given to its substrate AMP-morpholidate when different amounts of

inhibitor **19b** were present. The results showed that slight inhibition of Hint1 was already achieved when a ratio of 1:10 of Hint1 to compound **19b** was used. At a ratio of 1:1000, however, the degree of inhibition appeared similar to the Fhit/Ap<sub>3</sub>A experiment (**Figure 56**).

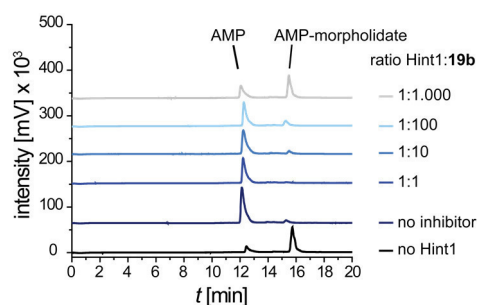


**Figure 56:** Result of an analytical HPLC assay analyzing the cleavage of AMP-morpholidate by Hint1 when increasing amounts of inhibitor **19b** were applied.

These results showed that although highly potent Fhit inhibitors were identified by high-throughput screening that only show moderate cytotoxicity in human cells, the developed compounds might be also inhibitors for other members of the Hit family. To date, the whole enzyme family has not been comprehensively studied so these inhibitors might also contribute to a better understanding of not only Fhit metabolism and its tumor suppressor activity, but also to a deeper knowledge of the other Hit proteins and their biological functions.

## 2.6 Discussion and Outlook

In summary, the high-throughput screening of small molecule compounds towards inhibition of the enzymatic activity of the tumor suppressor Fhit delivered several highly potent hits. Various derivatives were synthesized from two clusters identified in the screening, analyzed in dose-response assays and revealed even more effective inhibitors. This is the first time that highly potent small molecule inhibitors were developed for Fhit that significantly differ from the Ap<sub>3</sub>A structure.

Small molecule inhibitors of Fhit are of particular interest since experiments with inactive mutants or small interference RNA (siRNA) suffer from several drawbacks like differing phenotypes, experimental setups over several days or limitations due to cell type or transfection efficiencies. The developed compounds exert fast target inhibition and since they bind reversibly, they can even be applied in dynamic studies. Furthermore, the wild-type enzyme can be employed which does not alter the cellular surrounding in contrast to an absent protein.

The synthesized inhibitors have great application potential in research on Fhit's biological function, albeit their specificity against Fhit must be evaluated in further detail. As briefly mentioned within this thesis, the most potent compound also influenced the enzymatic activity of Hint1, whereas the other members of the Hit family have not been tested. X-ray

crystallography could indicate which further modifications are tolerated and display the differences in the various binding modes of the Hit family members. On this grounds, also small molecules can be developed that inhibit each Hit family member specifically.

Non-hydrolyzable  $Ap_nA$  analogues as Fhit inhibitors have already been applied in studies concerning Fhit-dependent and caspase-directed apoptosis. Hence, it will be interesting to investigate the developed compounds with respect to apoptotic signaling. Since the inhibitors are only moderately to non-toxic in two human cell lines tested they are ideally suited for these studies. When a prolongation of the signal's lifetime is detected, the newly identified compounds might also be interesting in the future development of anti-cancer drugs in Fhit-positive cells.



## IV Summary

Despite being present in all organisms and various cell types, still little is known about the small molecule  $\text{Ap}_3\text{A}$ . Discovered in the 1960's its appreciation varied between ATP surrogate, alarmone, second messenger and waste product over the years. Although observed intra- and extracellularly in multiple signaling pathways, designated physiological functions as well as a detailed mechanism of action remain to be investigated. Additionally, a defining role of  $\text{Ap}_3\text{A}$  with respect to other members of the  $\text{Ap}_n\text{A}$  family needs to be determined.

Interaction partners are particularly important in the elucidation of signal transduction pathways and in obtaining information about the fate of small molecules in a cellular surrounding. The Fhit protein hydrolyses  $\text{Ap}_3\text{A}$  and is the only known and studied interaction partner of  $\text{Ap}_3\text{A}$  so far. It is part of the Hit-family of proteins and expressed from the *FHIT* gene, one of the most frequently altered genes in the human genome. Hence, the disturbance of regular Fhit expression is an early event in cancer development.<sup>173</sup> Upon re-expression, malignancies are prevented by triggering apoptosis in abnormal cells.<sup>182-184</sup> Exact mechanisms of this tumor suppressor activity in complex with  $\text{Ap}_3\text{A}$ , however, remain to be elucidated.

The aim of this thesis was the development of chemical tools for the investigation of  $\text{Ap}_3\text{A}$ . Thus, a photoreactive affinity-based proteomic profiling (ABPP) probe was developed and utilized in human cell lysate to identify the interactome of  $\text{Ap}_3\text{A}$  with quantitative label-free mass spectrometry. For the first time,  $\text{Ap}_3\text{A}$  was the subject of investigation in this respect. Furthermore, small molecule inhibitors of the enzymatic activity of Fhit were identified by high-throughput screening and further improved by synthesis of derivatives.

In the first part of the thesis, a novel  $\text{Ap}_3\text{A}$  analogue was designed, synthesized and utilized in competitive photoreactive ABPP assays in human cell lysates (**Figure 57**). Probe C comprised an affinity tag and a photoaffinity group, able to undergo photoreactions and form covalent linkages to proteins in close proximity. Moreover, the phosphate bridges were synthesized to be non-hydrolyzable for higher stability in cell lysates.

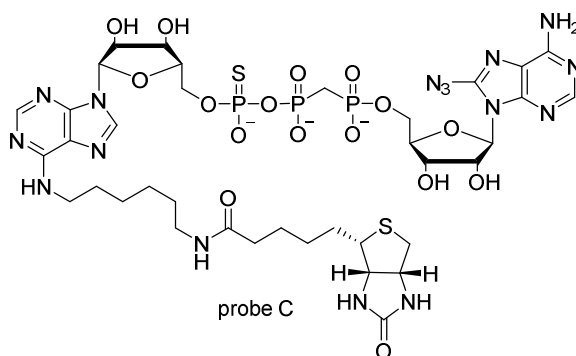


**Figure 57:** Molecular structure of probe C.

The photoreactive ABPP experiments were carried out competitively with a non-modified non-hydrolyzable Ap<sub>3</sub>A analogue and multiple proteins were identified that have not been associated with Ap<sub>3</sub>A or other members of the Ap<sub>n</sub>A family before. It was further ascertained that the respective proteins did not bind Ap<sub>3</sub>A as an ATP surrogate. These results point towards a distinct role of Ap<sub>3</sub>A with respect to ATP and other Ap<sub>n</sub>As. Further analysis revealed that the majority of identified proteins has not yet been linked to nucleotide binding in general. Indeed, many of these proteins are still uncharacterized regarding their functionalities. Thus, additional characteristics for Ap<sub>3</sub>A recognition, binding or processing are highly probable.

In the second part of this thesis, a high-throughput screening of small molecule libraries containing 15.136 compounds was performed and 45 hits were identified that inhibited Fhit's enzymatic cleavage of a FRET-labeled Ap<sub>3</sub>A up to at least 90%. Upon clustering, two main motifs were obtained that were derivatized in order to reveal even more potent inhibitors. On this basis, 18 compounds were synthesized, several thereof described for the first time, through microwave-assisted one-pot synthesis. The developed compounds were tested in *in vitro* dose-response assays for their inhibitory potential for Fhit's enzymatic activity. The best hit compound **19b** exhibited a highly potent *IC*<sub>50</sub> value of  $49 \pm 7$  nM (**Figure 58**).

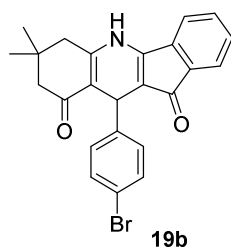


**Figure 58:** Molecular structure of the most potent Fhit inhibitor **19b**, identified in this study.

The synthesized small molecule inhibitors were tested for their cytotoxic potential in Fhit-positive HEK 293T and Fhit-negative HI299 cells. HEK 293T cells were more affected than HI299 cells, overall, a moderate to non-toxic potential towards these cell lines was revealed. The validity of the inhibitory potential of the obtained compounds was confirmed by application of **19b** in a system with natural Ap<sub>3</sub>A, in which Fhit was also inhibited. Further characterization showed that **19b** bound reversibly to Fhit and, interestingly, also inhibited the enzymatic activity of Hit family member Hint1.

The developed chemical tools significantly expand the prospects for studying Ap<sub>3</sub>A in human cells. Once validated, the identified interacting proteins enable a more detailed analysis of Ap<sub>3</sub>A's function in various signal transduction pathways and might contribute to the detection of a novel physiological role for the whole Ap<sub>n</sub>A family. The developed inhibitors have high potential in future investigations of Fhit and might unravel the intricate mechanism of its tumor suppressor functionality in complex with Ap<sub>3</sub>A. Besides, some of the identified proteins from ABPP might be involved in Fhit-induced signal transduction and can therefore contribute to the elucidation of Ap<sub>3</sub>A's role in this context.

## V Zusammenfassung

Trotz seines Vorkommens in sämtlichen Organismen und verschiedenen Zelltypen, ist noch immer wenig über das Molekül  $\text{Ap}_3\text{A}$  bekannt. Nach seiner Entdeckung in den 1960er Jahren, variierte das Interesse über die Verbindung über die Jahre zwischen ATP-Ersatz, Alarmon, sekundärem Botenstoff und Abfallprodukt. Obwohl es intra- wie auch extrazellulär in verschiedenen Signalwegen gefunden werden konnte, müssen exakte physiologische Funktionen als auch ein detaillierter Wirkmechanismus noch untersucht werden. Zusätzlich sollte  $\text{Ap}_3\text{A}$  auch eine bestimmte Rolle zugewiesen werden, besonders im Hinblick auf weitere Mitglieder der  $\text{Ap}_n\text{A}$  Familie.

Interaktionspartner spielen bei der Aufklärung von Signaltransduktionswegen sowie beim Sammeln von Informationen über den Verbleib von Molekülen im zellulären Umfeld eine wichtige Rolle. Das Fhit Protein hydrolysiert  $\text{Ap}_3\text{A}$  und ist bislang das einzig bekannte und untersuchte Interaktionsprotein von  $\text{Ap}_3\text{A}$ . Es ist Teil der Hit Proteinfamilie und wird vom *FHIT*-Gen exprimiert, eines der am häufigsten veränderten Gene im humanen Genom. Daher kommt die Störung der Fhit-Expression in frühen Stadien der Krebsentwicklung vor. Durch Re-Expression können Bösartigkeiten durch die Auslösung von Apoptose in abnormalen Zellen verhindert werden. Die Aufklärung dieser tumorsuppressiven Aktivität im Komplex mit  $\text{Ap}_3\text{A}$  muss allerdings noch abschließend untersucht werden.

Das Ziel dieser Arbeit war die Entwicklung von chemischen Werkzeugen für die Untersuchung von  $\text{Ap}_3\text{A}$ . Dafür wurde eine photoreaktive ABPP Sonde entwickelt und in humanem Zelllysats eingesetzt, um das Interaktom von  $\text{Ap}_3\text{A}$  mittels quantitativer markierungsfreier Massenspektrometrie zu identifizieren. Diesbezüglich war dabei zum ersten Mal  $\text{Ap}_3\text{A}$  Gegenstand der Untersuchung. Des Weiteren wurden Inhibitoren der enzymatischen Aktivität von Fhit durch ein Hochdurchsatz-Screening von Molekülbibliotheken identifiziert und durch Synthese von Derivaten weiterentwickelt und verbessert.

Im ersten Teil der vorliegenden Arbeit wurde ein neues  $\text{Ap}_3\text{A}$  Analog entworfen, synthetisiert und in kompetitiven photoreaktiven ABPP Experimenten in humanen Zelllysaten eingesetzt. Sonde C enthielt eine Affinitätsmarkierung sowie eine Photoaffinitätsfunktionalität, um damit Photoreaktionen und kovalente Bindungen zu Proteinen in nächster Nähe einzugehen. Weiterhin wurden die Phosphatbrücken so modifiziert, dass sie nicht hydrolysierbar waren, um eine höhere Stabilität in Zelllysaten zu gewährleisten.

Die photoreaktiven ABPP Experimente wurden kompetitiv mit einem unmodifizierten und nicht hydrolysierbaren  $\text{Ap}_3\text{A}$  Analog durchgeführt und viele Proteine wurden identifiziert, die bislang nicht mit  $\text{Ap}_3\text{A}$  oder anderen Mitgliedern der  $\text{Ap}_n\text{A}$  Familie in Verbindung gebracht wurden. Es wurde weiterhin herausgefunden, dass die betreffenden Proteine nicht als ATP-

Ersatzstoff gebunden wurden. Diese Ergebnisse deuten auf eine eigenständige Rolle des Ap<sub>3</sub>As bezüglich ATP und anderen Ap<sub>n</sub>As hin. Weitere Analysen zeigten, dass die Mehrheit der potentiellen Interaktionspartner bislang nicht mit Nukleotidbindung im Allgemeinen in Zusammenhang gebracht werden konnte. Tatsächlich konnte vielen dieser Proteine noch immer keine Funktionalität zugeordnet werden. Aus diesem Grund sind zusätzliche Eigenschaften für Ap<sub>3</sub>A-Erkennung, -Bindung und -Verarbeitung sehr wahrscheinlich.

In zweiten Teil dieser Arbeit wurde ein Hochdurchsatz-Screening von Molekül-Bibliotheken mit 15.136 Verbindungen durchgeführt und 45 Hits wurden identifiziert, die Fhit's enzymatische Spaltung eines FRET-markierten Ap<sub>3</sub>As bis zu mindestens 90% inhibierten. Durch Clusterbildung konnten zwei Hauptmotive identifiziert werden, die weiter derivatisiert wurden, um noch potentere Inhibitoren zu erhalten. Auf dieser Basis wurden 18 Verbindungen durch Microwellen-gestützte Eintopfsynthese synthetisiert, einige davon zum ersten Mal. Die entwickelten Verbindungen wurden in *in vitro* Dosis-Wirkungs-Experimenten auf ihr inhibitorisches Potential gegenüber Fhit's enzymatischer Aktivität getestet. Der beste Hit Verbindung **19b** wies einen sehr potenten IC<sub>50</sub>-Wert von 49 ± 7 nM auf.

Die synthetisierten Inhibitoren wurden weiterhin auf ihr zytotoxisches Potential in Fhit-positiven HEK 293T und Fhit-negativen HI299 Zellen getestet. Dabei waren HEK 293T Zellen sensibler gegenüber den Inhibitoren als HI299, allgemein wurde ein moderates bis nicht-toxisches Potential gegenüber diesen Zelllinien ermittelt. Die Aussagekraft des inhibitorischen Potentials der erhaltenen Verbindungen wurde durch die Anwendung von **19b** in einem System mit natürlichem Ap<sub>3</sub>A bestätigt, in welchem Fhit ebenfalls inhibiert wurde. Weitere Analysen zeigten, dass Verbindung **19b** reversibel an Fhit gebunden wurde und interessanterweise auch die enzymatische Aktivität des Hit Familienmitglieds Hint1 inhibierte.

Die entwickelten chemischen Werkzeuge erweitern signifikant die Möglichkeiten für die Untersuchung von Ap<sub>3</sub>A in humanen Zellen. Nach der Validierung, ermöglichen die identifizierten Interaktionsproteine eine detailliertere Analyse der Funktionen von Ap<sub>3</sub>A in verschiedenen Signaltransduktionswegen und können möglicherweise dazu beitragen eine neue physiologische Rolle der ganzen Ap<sub>n</sub>A Familie zu entdecken. Die entwickelten Inhibitoren haben großes Potential in der zukünftigen Untersuchung von Fhit und könnten den komplizierten Mechanismus seiner tumorsuppressiven Aktivität im Komplex mit Ap<sub>3</sub>A aufdecken. Zudem könnten einige der identifizierten Proteine aus den ABPP Experimenten in Fhit-induzierten Signalwegen involviert sein und so die Rolle von Ap<sub>3</sub>A in diesem Zusammenhang weiter aufklären.

## VI Experimental Section

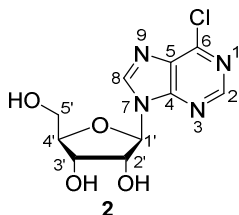
### 1 Chemical Syntheses

#### 1.1 General Information

All reagents were commercially available and used without further purification. Solvents were used as p.a. grade, purified by distillation as needed and dry solvents were purchased from *Sigma Aldrich*. TMP and  $\text{PSCl}_3$  were freshly distilled under nitrogen atmosphere. If necessary, reactions were conducted under the exclusion of air or moisture. Counterions are not indicated in structures and NMR for simplicity. Anion exchange chromatography was performed on an ÄktaPurifier (*GE Healthcare*) with a DEAE Sephadex<sup>TM</sup> A-25 (*GE Healthcare*) column using a linear gradient of 0.1 M to 1.0 M of triethylammonium bicarbonate buffer (TEAB, pH 7.5). RP-HPLC was performed using *Shimadzu* systems equipped with Prominence LC 20AP (preparative) or Prominence LC 10AT (analytical) pumps and SPD M20A PDA detectors. Preparative separations were conducted on 250/21 (15 mL/min) or 250/16 (8 mL/min) Nucleodur C18 HTec, 5  $\mu\text{m}$  columns (*Macherey-Nagel*) and gradients of MeCN in 50 mM triethylammonium acetate buffer (TEAA, 50 mM, pH 7.0) were used and adjusted accordingly. All compounds purified by RP-HPLC were obtained as their triethylammonium salts after repeated freeze-drying (VaCo 2-II-E (*Zirbus*), Lyovac GT2 (*Leybold-Heraeus*), Beta 2-8 LDplus (*Christ*)). MPLC was performed on a Sepacore X50 (*Büchi*) instrument using SVF D22 RP-18 columns (*Götec Labortechnik*) with gradients of MeCN in  $\text{H}_2\text{O}$ . Thin layer chromatography (TLC) was performed using aluminum sheets with silica gel 60 F<sub>254</sub> and reversed phase silica gel 60 RP-18 F<sub>254S</sub> (*Merck*). NMR spectra were recorded on Avance III 400 MHz (*Bruker*). <sup>1</sup>H, <sup>13</sup>C, <sup>19</sup>F and <sup>31</sup>P NMR chemical shifts are reported relative to the residual solvent peak and are given in ppm ( $\delta$ ). s: singlet, d: doublet, t: triplet, q: quartet, m: multiplet. Coupling constants *J* are given in Hz. High-resolution MS was performed on a micrOTOF II (*Bruker*), coupled to a 1260 Infinity HPLC system (*Agilent*). Microwave syntheses were conducted on a Monowave 300 system (*Anton Paar*). The reported yields refer to analytically pure substances and were obtained via UV absorption with the respective reported absorption coefficients in case of phosphates. All temperatures quoted are uncorrected.

## 1.2 Syntheses Towards the Different ABPs

### 1.2.1 Synthesis of 6-Chloroinosine (2)



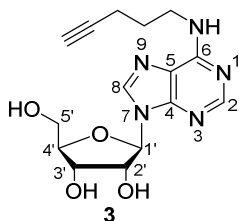
Inosine (4.00 g, 14.9 mmol, 1.0 eq.) was suspended in DCM (100 mL) and trifluoroacetic anhydride (34.4 g, 32.1 mL, 164 mmol, 11.0 eq.) was added. The reaction mixture was stirred at rt for 16 h. The solvents were carefully evaporated under reduced pressure and the residue was dissolved in DCM (500 mL). The solution was cooled to 0 °C and a mixture of DMF (2.89 g, 3.04 mL, 38.7 mmol, 2.6 eq.) and thionyl chloride (9.94 g, 6.06 mL, 83.6 mmol, 5.6 eq.) in DCM (160 mL) was added dropwise. The solution was refluxed for 20 h. The mixture was cooled to rt, concentrated to 200 mL and washed with saturated sodium hydrogencarbonate solution (200 mL). The aqueous layer was extracted with DCM (2 x 150 mL). The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue was dissolved in MeOH (8 mL) and refluxed for 16 h. Et<sub>2</sub>O (30 mL) was added to complete the precipitation. The product **2** (2.75 g, 9.59 mmol, 64%) was collected by filtration and drying *in vacuo* as a white solid.

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) = 8.95 (s, 1H, H-8), 8.82 (s, 1H, H-2), 6.05 (d, *J* = 5.3 Hz, 1H, H-1'), 5.57 (d, *J* = 5.8 Hz, 1H, 2'-OH), 5.25 (d, *J* = 5.2 Hz, 1H, 3'-OH), 5.09 (t, *J* = 5.5 Hz, 1H, 5'-OH), 4.60 (q, *J* = 5.3 Hz, 1H, H-2'), 4.20 (q, *J* = 4.9 Hz, 1H, H-3'), 4.00 (q, *J* = 3.9 Hz, 1H, H-4'), 3.75–3.68 (m, 1H, H-5'<sub>a</sub>), 3.63–3.57 (m, 1H, H-5'<sub>b</sub>).

<sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) = 152.2 (C-6), 152.1 (C-2), 149.8 (C-5), 146.2 (C-8), 131.9 (C-4), 88.6 (C-1'), 86.2 (C-4'), 74.4 (C-2'), 70.4 (C-3'), 61.5 (C-5').

HR-ESI-MS (*m/z*): 287.0535 [M+H]<sup>+</sup> (calc. 287.0542 [M+H]<sup>+</sup>, C<sub>10</sub>H<sub>12</sub>ClN<sub>4</sub>O<sub>4</sub><sup>+</sup>), deviation: 2.43 ppm.

### 1.2.2 Synthesis of *N*6-(Pent-4-yn-1-yl)adenosine (3)



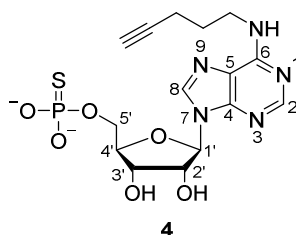
6-Chloroinosine (840 mg, 293  $\mu$ mol, 1.0 eq.) and 1-amino-4-pentyne (730 mg, 8.78 mmol, 3.0 eq.) were suspended in EtOH (10 mL) and heated to 85 °C for 4 h. The reaction mixture was cooled to rt and stirred for another 12 h to complete precipitation. The product **3** (840 mg, 252  $\mu$ mol, 86%) was collected by filtration and drying *in vacuo* as a white solid.

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  (ppm) = 8.34 (s, 1H, H-8), 8.21 (s, 1H, H-2), 7.91 (s<sub>br</sub>, 1H, NH), 5.88 (d, *J* = 6.2 Hz, 1H, H-1'), 5.41 (d, *J* = 6.2 Hz, 1H, 2'OH), 5.38 (dd, *J* = 7.1, 4.5 Hz, 1H, 5'OH), 5.16 (d, *J* = 4.5 Hz, 1H, 3'OH), 4.61 (q, *J* = 5.8 Hz, 1H, H-2'), 4.14 (dd, *J* = 4.8, 3.0 Hz, 1H, H-3'), 3.96 (q, *J* = 3.5 Hz, 1H, H-4'), 3.67 (dt, *J* = 12.0, 4.1 Hz, 1H, H-5'<sub>a</sub>), 3.55 (ddd, *J* = 12.0, 7.1, 3.5 Hz, 2H, H-5'<sub>b</sub>, C6-NH-CH<sub>2</sub>), 2.77 (t, *J* = 2.7 Hz, 1H, C-CH), 2.23 (dt, *J* = 7.2, 2.6 Hz, 2H, CH-C-CH<sub>2</sub>), 1.79 (p, *J* = 7.2 Hz, 2H, C6-NH-CH<sub>2</sub>-CH<sub>2</sub>).

<sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  (ppm) = 160.5 (C-6), 152.3 (C-2), 139.7 (C-8), 134.0 (C-4), 114.9 (C-5), 87.9 (C-1'), 85.9 (C-4'), 84.1 (HC-C), 73.5 (C-2'), 71.3 (HC-C), 70.6 (C-3'), 61.6 (C-5'), 28.0 (C6-NH-CH<sub>2</sub>-CH<sub>2</sub>), 15.4 (HC-C-CH<sub>2</sub>).

HR-ESI-MS (*m/z*): 334.1514 [M+H]<sup>+</sup> (calc. 334.1510 [M+H]<sup>+</sup>, C<sub>15</sub>H<sub>20</sub>N<sub>5</sub>O<sub>4</sub><sup>+</sup>), deviation: 1.20 ppm.

### 1.2.3 Synthesis of N6-(Pent-4-yn-1-yl)adenosine 5'-Thiophosphate (**4**)



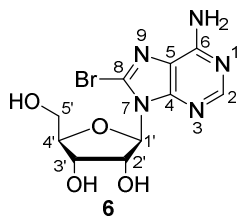
N6-(Pent-4-yn-1-yl)adenosine (150 mg, 450  $\mu$ mol, 1.0 eq.) and proton sponge (190 mg, 900  $\mu$ mol, 2.0 eq.) were dried *in vacuo* for 30 min, dissolved in TMP (6 mL) and cooled to 0 °C. PSCl<sub>3</sub> (75.9 mg, 46.0  $\mu$ L, 495  $\mu$ mol, 1.1 eq.) was added dropwise and the solution was stirred at 0 °C for 1 h. The reaction was stopped by addition of TEAB buffer (0.2 M, pH = 7, 15 mL) and the solution was stirred at rt for another 30 min. The reaction mixture was washed with EtOAc (3 x 25 mL) and the aqueous phase was concentrated and purified by anion-exchange chromatography and RP-HPLC. The product **4** (243  $\mu$ mol, 54%) was obtained as a white solid.

<sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O):  $\delta$  (ppm) = 8.64 (s, 1H, H-8), 8.25 (s, 1H, H-2), 6.14 (d, *J* = 6.1 Hz, 1H, H-1'), 4.83 (t, *J* = 5.5 Hz, 1H, H-2'), 4.58–4.53 (m, 1H, H-3'), 4.44–4.38 (m, 1H, H-4'), 4.16–4.06 (m, 2 H, H-5'<sub>a,b</sub>), 3.80–3.60 (m, 2H, C6-NH-CH<sub>2</sub>), 2.41–2.32 (m, 3H, CH-C-CH<sub>2</sub>, HC-C), 1.96–1.90 (m, 2H, C6-NH-CH<sub>2</sub>-CH<sub>2</sub>).

<sup>31</sup>P NMR (160 MHz, D<sub>2</sub>O):  $\delta$  (ppm) = 44.63 (s, 1H, Ps).

HR-ESI-MS (*m/z*): 428.0789 [M-H]<sup>-</sup> (calc. 429.0788 [M-H]<sup>-</sup>, C<sub>15</sub>H<sub>19</sub>N<sub>5</sub>O<sub>6</sub>PS<sup>-</sup>), deviation: 0.24 ppm.

### 1.2.4 Synthesis of 8-Bromoadenosine (6)



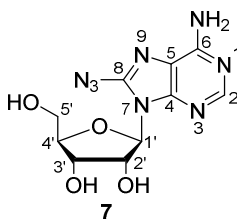
Saturated bromine-water (2.4 mL Br<sub>2</sub> in 200 mL) was added slowly to a suspension of adenosine (5.00 g, 18.7 mmol, 1.0 eq.) in NaOAc buffer (150 mL, 0.5 M, pH = 4). The mixture was stirred at rt for 47 h. The solution was decolorized by the addition of NaHSO<sub>3</sub> (60 mL, 5 M) and the pH of the solution was adjusted to 7 with NaOH (100 mL, 2 M). The precipitate was collected by filtration, successively washed with water (3 x 50 mL) and acetone (2 x 50 mL) and dried *in vacuo*. The product **6** (4.03 g, 11.6 mmol, 62%) was yielded as a white solid.

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) = 8.12 (s, 1H, H-2), 7.57 (s<sub>br</sub>, 2H, NH<sub>2</sub>), 5.84 (d, *J* = 6.8 Hz, 1H, H-1'), 5.50 (dd, *J* = 8.6, 3.9 Hz, 1H, 5'-OH), 5.46 (d, *J* = 6.3 Hz, 1H, 2'-OH), 5.23 (d, *J* = 4.5 Hz, 1H, 3'-OH), 5.10 (q, *J* = 6.4 Hz, 1H, H-2'), 4.22–4.19 (m, 1H, H-3'), 4.00–3.97 (m, 1H, H-4'), 3.71–3.66 (m, 1H, H-5'<sub>a</sub>), 3.56–3.50 (m, 1H, H-5'<sub>b</sub>).

<sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) = 155.6 (C-8), 152.9 (C-2), 150.3 (C-6), 127.6 (C-5), 120.1 (C-4), 90.9 (C-1'), 87.2 (C-4'), 71.6 (C-2'), 71.3 (C-3'), 62.6 (C-5').

HR-ESI-MS (*m/z*): 346.0157 [M+H]<sup>+</sup> (calc. 346.0145 [M+H]<sup>+</sup>, C<sub>10</sub>H<sub>13</sub>BrN<sub>5</sub>O<sub>4</sub><sup>+</sup>), deviation: 3.47 ppm.

### 1.2.5 Synthesis of 8-Azidoadenosine (7)



8-Bromoadenosine (1.50 g, 4.33 mmol, 1.0 eq.) and sodium azide (900 mg, 13.8 mmol, 3.2 eq.) were dissolved in dry DMF (12 mL) and heated to 60 °C. After 24 h additional sodium azide (8.66 mmol, 2.0 eq.) was added and the solution was stirred for 3 h. The solvent was evaporated to dryness. The resulting product **7** (706 mg, 2.29 mmol, 53%) was recrystallized from water (8 mL) as an orange solid.

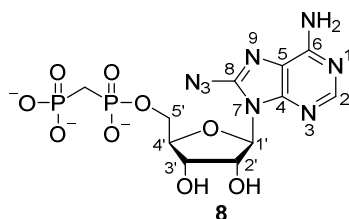
<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) = 8.08 (s, 1H, H-2), 7.32 (s<sub>br</sub>, 2H, NH<sub>2</sub>), 5.64 (d, *J* = 6.6 Hz, 1H, H-1'), 5.42–5.36 (m, 2H, 2'-OH, 5'-OH), 5.19–5.14 (m, 1H, 3'-OH), 4.93–4.87 (m,

<sup>1</sup>H, H-2'), 4.18–4.14 (m, 1H, H-3'), 3.95–3.91 (m, 1H, H-4'), 3.70–3.62 (m, 1H, H-5'<sub>a</sub>), 3.56–3.47 (m, 1H, H-5'<sub>b</sub>).

<sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  (ppm) = 154.9 (C-6), 152.1 (C-2), 149.8 (C-4), 144.9 (C-8), 117.5 (C-5), 88.1 (C-1'), 86.8 (C-4'), 71.7 (C-2'), 71.3 (C-3'), 62.6 (C-5').

HR-ESI-MS (*m/z*): 309.1056 [M+H]<sup>+</sup> (calc. 309.1054 [M+H]<sup>+</sup>, C<sub>10</sub>H<sub>13</sub>N<sub>8</sub>O<sub>4</sub><sup>+</sup>), deviation: 0.65 ppm.

### 1.2.6 Synthesis of 8-Azidoadenosine 5'-Methylene Bisphosphonate (**8**)



At 0 °C a solution of methylene bis(phosphonic dichloride) (478 mg, 1.91 mmol, 3.0 eq.) was dissolved in TMP (10 mL) and given to a solution of 8-azidoadenosine (200 mg, 649  $\mu$ mol, 1.0 eq.) in TMP (9 mL). The reaction mixture was stirred for 3.5 h at 0 °C. The reaction was stopped by addition of TEAB buffer (0.2 M, pH = 7, 15 mL) and stirring for 30 min at rt. The solution was washed with EtOAc (3 x 50 mL) and the aqueous phase was concentrated and purified by anion-exchange chromatography and RP-HPLC. The product **8** as its triethylammonium salt (341  $\mu$ mol, 53%) was obtained as a white solid.

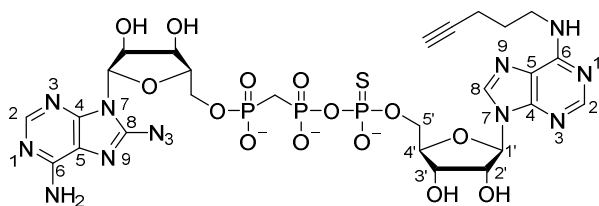
<sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O):  $\delta$  (ppm) = 7.91 (s, 1H, H-2), 5.73 (d, *J* = 5.5 Hz, 1H, H-1'), 4.85 (t, *J* = 5.6 Hz, 1H, H-2'), 4.40 (dd, *J* = 5.6, 4.4 Hz, 1H, H-3'), 4.10–3.94 (m, 3H, H-4', H-5'<sub>a</sub>, H-5'<sub>b</sub>), 2.01 (t, *J* = 19.8 Hz, 2H, CH<sub>2</sub>).

<sup>13</sup>C NMR (100 MHz, MeOD):  $\delta$  (ppm) = 155.6 (C-6), 153.0 (C-2), 151.6 (C-4), 147.1 (C-8), 118.6 (C-5), 89.1 (C-1'), 85.3 (C-4'), 72.7 (C-2'), 72.2 (C-3'), 65.5 (C-5'), 29.1 (CH<sub>2</sub>).

<sup>31</sup>P NMR (160 MHz, D<sub>2</sub>O):  $\delta$  (ppm) = 18.21 (d, *J* = 9.1 Hz, P <sub>$\alpha$</sub> ), 14.69 (d, *J* = 9.1 Hz, P <sub>$\beta$</sub> ).

HR-ESI-MS (*m/z*): 465.0441 [M-H]<sup>-</sup> (calc. 465.0432 [M-H]<sup>-</sup>, C<sub>11</sub>H<sub>15</sub>N<sub>8</sub>O<sub>9</sub>P<sub>2</sub><sup>-</sup>), deviation: 1.94 ppm.

## 1.2.7 Synthesis of Probe A



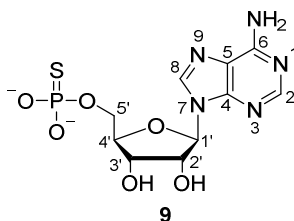
*N*6-(Pent-4-yn-1-yl)adenosine 5'-thiomonophosphate (100 mg, 188  $\mu$ mol, 1.0 eq.) was dissolved in dry MeOH (7 mL) and *N*-octylamine (69.7 mg, 86.0  $\mu$ L, 197  $\mu$ mol, 1.05 eq.) was added. The solution was shaken and evaporated under reduced pressure. The residue was dissolved in dry pyridine (3 x 10 mL), evaporated again under reduced pressure and dried over  $P_2O_5$  overnight. The residue was dissolved in dioxane (3 mL) and diphenylphosphoryl chloride (77.3 mg, 59.6  $\mu$ L, 288  $\mu$ mol, 1.53 eq.) and tributylamine (83.6 mg, 107  $\mu$ L, 451  $\mu$ mol, 2.4 eq.) were added. The solution was stirred at rt for 3.5 h and evaporated under reduced pressure. Diethylether (3 x 10 mL) was added, the flask shaken and the solvent removed with a syringe. The residue was dissolved in pyridine (2 x 10 mL) and evaporated under reduced pressure. 8-Azidoadenosine 5'-methylenediphosphate (82.0 mg, 131  $\mu$ mol, 0.7 eq.) was dissolved in dry MeOH (5 mL) and tributylamine (53.4 mg, 68.4  $\mu$ L, 288  $\mu$ mol, 1.53 eq.) was added. The solution was shaken and evaporated under reduced pressure. The residue was dissolved in dry pyridine (3 x 10 mL), evaporated again under reduced pressure and dried over  $P_2O_5$  overnight. The residue was dissolved in dry pyridine (4.5 mL) and given to the solution of the activated thiophosphate. The reaction mixture was stirred at rt overnight. The solvents were evaporated under reduced pressure, the residue dissolved in water (50 mL) and extracted with DCM (2 x 20 mL). The aqueous phase was concentrated and purified by anion-exchange chromatography and RP-HPLC. The product (13 mg, 11.0  $\mu$ mol, 6%) was obtained as a diastereomeric mixture. The diastereoisomers were successfully separated by further RP-HPLC.

$^1H$  NMR (400 MHz,  $D_2O$ ):  $\delta$  (ppm) = 8.37 (s, 1H, H-8), 8.07 (s, 1H, H-2<sub>a</sub>), 8.04 (s, 1H, H-2<sub>b</sub>), 5.95 (d,  $J$  = 5.2 Hz, 1H, H-1'<sub>a</sub>), 5.75 (d,  $J$  = 6.1 Hz, 1H, H-1'<sub>b</sub>), 4.88 (t,  $J$  = 5.8 Hz, 1H, H-2'<sub>b</sub>), 4.55 (t,  $J$  = 6.1 Hz, 1H, H-2'<sub>a</sub>), 4.49–4.44 (m, 2H), 4.35–4.30 (m, 1H), 4.29–4.22 (m, 2H), 4.21–4.09 (m, 3H), 3.53 (s<sub>br</sub>, 2H, C6-NH-CH<sub>2</sub>), 2.49–2.33 (m, 2H), 2.33–2.24 (m, 3H), 1.82 (p,  $J$  = 7.0 Hz, 2H, C6-NH-CH<sub>2</sub>-CH<sub>2</sub>).

$^{31}P$  NMR (160 MHz,  $D_2O$ ):  $\delta$  (ppm) = 42.98–42.61 (m, 1P, P<sub>S</sub>), 17.23–16.91 (m, 1P, P <sub>$\alpha$</sub> ), 7.52–7.12 (m, 1P, P <sub>$\beta$</sub> ).

HR-ESI-MS ( $m/z$ ): 876.1208 [ $M-H$ ]<sup>−</sup> (calc. 876.1198 [ $M-H$ ]<sup>−</sup>, C<sub>26</sub>H<sub>33</sub>N<sub>3</sub>O<sub>14</sub>P<sub>3</sub>S<sup>−</sup>), deviation: 1.14 ppm.

### 1.2.8 Synthesis of Adenosine 5'-Thiophosphate (9)



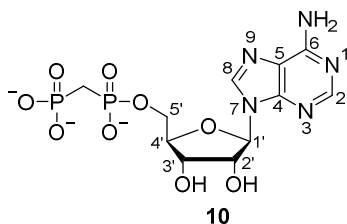
Adenosine (200 mg, 749  $\mu$ mol, 1.0 eq.) was dried *in vacuo* for 10 min, dissolved in dry pyridine (7 mL) and cooled to 0 °C. PSCl<sub>3</sub> (97.2 mg, 58.2  $\mu$ L, 574  $\mu$ mol, 1.2 eq.) was added dropwise and the solution was stirred at 0 °C for 1 h. The reaction was stopped by addition of TEAB buffer (0.2 M, pH = 7, 15 mL) and the solution was stirred at rt for another 30 min. The reaction mixture was concentrated under reduced pressure and purified by anion-exchange chromatography and RP-HPLC. The product **9** (337  $\mu$ mol, 45%) was obtained as a white solid.

<sup>1</sup>H NMR (400 MHz, MeOD):  $\delta$  (ppm) = 8.70 (s, 1H, H-8), 8.20 (s, 1H, H-2), 6.10 (d,  $J$  = 6.1 Hz, 1H, H-1'), 4.73 (t,  $J$  = 5.5 Hz, 1H, H-2'), 4.46 (dd,  $J$  = 5.0, 3.0 Hz, 1H, H-3'), 4.26–4.23 (m, 1H, H-4'), 4.20–4.08 (m, 2H, H-5'<sub>a</sub>, H-5'<sub>b</sub>).

<sup>31</sup>P NMR (160 MHz, MeOD):  $\delta$  (ppm) = 48.83 (s, 1P, P<sub>S</sub>).

HR-ESI-MS ( $m/z$ ): 362.0331 [M-H]<sup>-</sup> (calc. 362.0319 [M-H]<sup>-</sup>, C<sub>10</sub>H<sub>13</sub>N<sub>5</sub>O<sub>6</sub>PS<sup>-</sup>), deviation: 3.31 ppm.

### 1.2.9 Synthesis of Adenosine 5'-Methylene Bisphosphonate (10)



Adenosine (340 mg, 1.27 mmol, 1.0 eq.) and methylene bis(phosphonic) dichloride (953 mg, 3.82 mmol, 3.0 eq.) were separately dissolved in TMP (6 mL) and cooled to 0 °C. The methylene bis(phosphonic) dichloride solution was given to adenosine and the reaction mixture was stirred at 0 °C for 3.5 h. The reaction was stopped by addition of TEAB buffer (0.2 M, pH = 7, 20 mL) and stirring at rt for 30 min. The reaction mixture was extracted with EtOAc (3 x 25 mL) and the aqueous phase was concentrated and purified by anion-exchange chromatography and RP-HPLC. The product **10** (407 mg, 648  $\mu$ mol, 51%) was obtained as a white solid.

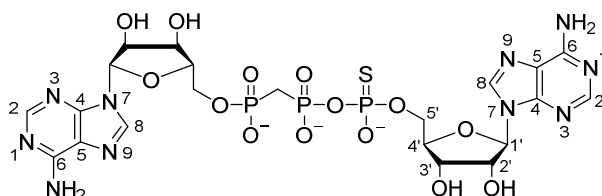
**<sup>1</sup>H NMR** (400 MHz, MeOD):  $\delta$  (ppm) = 8.57 (s, 1H, H-8), 8.20 (s, 1H, H-2), 6.09 (d,  $J$  = 5.4 Hz, 1H, H-1'), 4.71 (t,  $J$  = 5.3 Hz, 1H, H-2'), 4.52–4.46 (m, 1H, H-3'), 4.27–4.16 (m, 3H, H-4', H-5'a, H-5'b), 2.18 (t,  $J$  = 19.1 Hz, 2H, CH<sub>2</sub>).

**<sup>13</sup>C NMR** (100 MHz, MeOD):  $\delta$  (ppm) = 155.9 (C-6), 152.4 (C-2), 149.5 (C-4), 139.8 (C-8), 87.5 (C-1'), 84.2 (C-4'), 74.6 (C-2'), 70.6 (C-3'), 63.8 (C-5'), 27.7 (CH<sub>2</sub>).

**<sup>31</sup>P NMR** (160 MHz, MeOD):  $\delta$  (ppm) = 18.1 (P <sub>$\alpha$</sub> ), 14.2 (P <sub>$\beta$</sub> ).

**HR-ESI-MS** ( $m/z$ ): 424.0411 [M-H]<sup>-</sup> (calc. 424.0418 [M-H]<sup>-</sup>, C<sub>11</sub>H<sub>16</sub>N<sub>5</sub>O<sub>9</sub>P<sub>2</sub><sup>-</sup>), deviation: 1.65 ppm.

### 1.2.10 Synthesis of Probe B



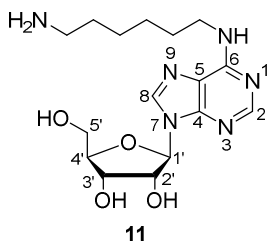
Adenosine methylene diphosphate (150  $\mu$ mol, 1.0 eq.) was co-evaporated with DMF (3 x 3 mL) and dried *in vacuo*. Imidazole (204 mg, 3.00 mmol, 20 eq.) and 2,2'-dithiopyridine (330 mg, 1.5 mmol, 10 eq.) were added and the reagents were suspended in dry DMF (5 mL). Triphenylphosphine (393 mg, 1.5 mmol, 10 eq.) and triethylamine (152 mg, 208  $\mu$ L, 1.5 mmol, 10 eq.) were added and the yellow solution was stirred at rt overnight. The mixture was given into a cold solution of sodium perchlorate (36.7 mg, 300  $\mu$ mol, 2.0 eq.) in acetone (25 mL) and the precipitate was collected by centrifugation (4000 rpm, 3 min) and washed with cold acetone (2 x 15 mL). The activated diphosphate (117  $\mu$ mol, 1.4 eq.) was dried over P<sub>2</sub>O<sub>5</sub> and dissolved in dry DMF (2 mL). Adenosine 5'-thiophosphate (83.6  $\mu$ mol, 1.0 eq.) and dry ZnCl<sub>2</sub> (182 mg, 1.34 mmol, 16 eq.) were added and the mixture was shaken until complete dissolution was achieved. The reaction mixture was stirred at rt for 6 d before it was quenched with EDTA (498 mg, 1.34 mmol, 16. eq.) in H<sub>2</sub>O (20 mL). Evaporation of the solvents, purification of the raw product by anion-exchange chromatography and RP-HPLC gave the product as a diastereomeric mixture (17.0  $\mu$ mol, 20%) that was separated by further RP-HPLC.

**<sup>1</sup>H NMR** (400 MHz, D<sub>2</sub>O):  $\delta$  (ppm) = 8.48 (s, 1H, H-8<sub>a</sub>), 8.43 (s, 1H, H-8<sub>b</sub>), 8.08 (d,  $J$  = 6.8 Hz, 2H, H-2<sub>a</sub>, H-2<sub>b</sub>), 5.89 (d,  $J$  = 5.1 Hz, 1H, H-1'<sub>a</sub>), 5.86 (d,  $J$  = 5.1 Hz, 1H, H-1'<sub>b</sub>), 4.57–4.50 (m, 2H, H-2'<sub>a</sub>, H-2'<sub>b</sub>), 4.42 (q,  $J$  = 4.6 Hz, 2H, H-3'<sub>a</sub>, H-3'<sub>b</sub>), 4.33–4.20 (m, 5H, H-4'<sub>a</sub>, H-4'<sub>b</sub>, H-5'<sub>a</sub>, H-5'<sub>b</sub>, H-5'<sub>c</sub>), 4.16–4.10 (m, 1H, H-5'<sub>d</sub>), 2.48 (tt,  $J$  = 21.1, 10.8 Hz, 2H, P-CH<sub>2</sub>-P).

**<sup>31</sup>P NMR** (160 MHz, D<sub>2</sub>O):  $\delta$  (ppm) = 42.9 (d,  $J$  = 32.3 Hz, 1P, P<sub>S</sub>), 17.4 (d,  $J$  = 7.7 Hz, 1P, P <sub>$\alpha$</sub> ), 7.57 (dd,  $J$  = 32.3, 7.7 Hz, 1P, P <sub>$\beta$</sub> ).

**HR-ESI-MS** ( $m/z$ ): 769.0715 (calc. 769.0715 [M-H]<sup>-</sup>, C<sub>21</sub>H<sub>28</sub>N<sub>10</sub>O<sub>14</sub>P<sub>3</sub>S<sup>-</sup>), deviation: 0.00 ppm.

### 1.2.11 Synthesis of N6-Aminohexyladenosine (11)



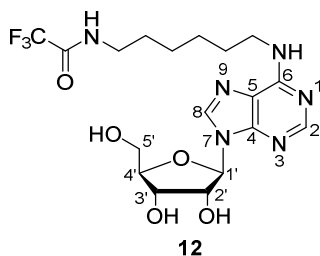
6-Chloroinosine (5.00 g, 17.4 mmol, 1.0 eq.) and 1,6-diaminohexane (6.10 g, 52.5 mmol, 3.) were suspended in EtOH (50 mL) and heated to reflux for 3.5 h. The reaction mixture was stirred at rt overnight and the precipitate was collected by filtration and washing with EtOH (3 x 50 mL). The product **11** (5.19 g, 14.2 mmol, 82%) was obtained as a white solid after drying *in vacuo*.

<sup>1</sup>H NMR (400 MHz, MeOD):  $\delta$  (ppm) = 8.24 (s, 1H, H-8), 8.21 (s<sub>br</sub>, 1H, H-2), 5.95 (d,  $J$  = 6.5 Hz, 1H, H-1'), 4.74 (dd,  $J$  = 6.5, 5.2 Hz, 1H, H-2'), 4.32 (dd,  $J$  = 5.2, 2.5 Hz, 1H, H-3'), 4.17 (q,  $J$  = 2.5 Hz, 1H, H-4'), 3.89 (dd,  $J$  = 12.6, 2.5 Hz, 1H, H-5<sub>a</sub>'), 3.74 (dd,  $J$  = 12.6, 2.5 Hz, 1H, H-5<sub>b</sub>'), 3.60 (s<sub>br</sub>, 2H, C6-NH-CH<sub>2</sub>), 2.63 (t,  $J$  = 7.0 Hz, 2H, NH<sub>2</sub>-CH<sub>2</sub>), 1.71 (p,  $J$  = 7.3 Hz, 2H, C6-NH-CH<sub>2</sub>-CH<sub>2</sub>), 1.53–1.36 (m, 6H, 3 x CH<sub>2</sub>-linker).

<sup>13</sup>C NMR (100 MHz, MeOD):  $\delta$  (ppm) = 160.5 (C-6), 152.1 (C-8), 142.6 (C-4), 140.0 (C-2), 125.9 (C-5), 89.9 (C-1'), 86.8 (C-4'), 74.0 (C-2'), 71.3 (C-3'), 62.1 (C-5'), 41.1 (C6-NH-CH<sub>2</sub>), 40.0 (NH<sub>2</sub>-CH<sub>2</sub>), 32.3 (CH<sub>2</sub>-linker), 26.4 (C6-NH-CH<sub>2</sub>-CH<sub>2</sub>), 26.3 (CH<sub>2</sub>-linker), 16.9 (CH<sub>2</sub>-linker).

**HR-ESI-MS** ( $m/z$ ): 367.2092 [M+H]<sup>+</sup> (calc. 367.2088 [M+H]<sup>+</sup>, C<sub>16</sub>H<sub>27</sub>N<sub>6</sub>O<sub>4</sub><sup>+</sup>), deviation: 1.09 ppm.

### 1.2.12 Synthesis of N6-Trifluoroacetamidohexyladenosine (12)



N6-Aminohexyladenosine (2.00 g, 5.50 mmol, 1.0 eq.) was suspended in MeOH (60 mL). NEt<sub>3</sub> (723 mg, 1.00 mL, 7.15 mmol, 1.3 eq.) and ethyl trifluoroacetate (1.20 g, 1.00 mL, 8.25 mmol, 1.5 eq.) were added and the reaction mixture was stirred at rt overnight. The solvent was

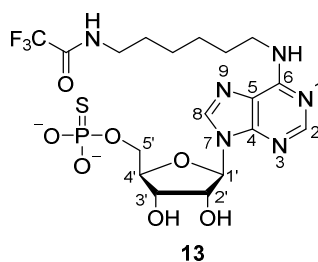
evaporated under reduced pressure and the crude product was purified by flash column chromatography on silica gel (DCM:MeOH, 10:1). The product **12** (2.10 g, 4.50 mmol, 81%) was obtained as a white solid after drying *in vacuo*.

**<sup>1</sup>H NMR** (400 MHz, MeOD):  $\delta$  (ppm) = 8.25 (s, 1H, H-8), 8.21 (s<sub>br</sub>, 1H, H-2), 5.95 (d,  $J$  = 6.4 Hz, 1H, H-1'), 4.74 (t,  $J$  = 5.7 Hz, 1H, H-2'), 4.32 (dd,  $J$  = 5.0, 2.5 Hz, 1H, H-3'), 4.19–4.16 (m, 1H, H-4'), 3.88 (dd,  $J$  = 12.6, 2.5 Hz, 1H, H-5'<sub>a</sub>), 3.74 (dd,  $J$  = 12.6, 2.5 Hz, 1H, H-5'<sub>b</sub>), 3.59 (s<sub>br</sub>, 2H, C6-NH-CH<sub>2</sub>), 3.28 (t,  $J$  = 7.2 Hz, 2H, NH<sub>2</sub>-CH<sub>2</sub>), 1.71 (p,  $J$  = 7.2 Hz, 2H, C6-NH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>), 1.59 (p,  $J$  = 7.2 Hz, 2H, CH<sub>2</sub>-linker), 1.47 (p,  $J$  = 7.2 Hz, 2H, CH<sub>2</sub>-linker), 1.44–1.38 (m, 2H, CH<sub>2</sub>-linker).

**<sup>13</sup>C NMR** (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  (ppm) = 156.1 (q,  $J$  = 35.6 Hz, COCF<sub>3</sub>), 154.6 (C-6), 152.4 (C-2), 148.2 (C-4), 139.6 (C-8), 119.7 (C-5), 116.0 (q,  $J$  = 289.3 Hz, CF<sub>3</sub>), 87.9 (C-1'), 85.9 (C-4'), 73.4 (C-2'), 70.7 (C-3'), 61.7 (C-5'), 28.9 (CH<sub>2</sub>), 28.2 (CH<sub>2</sub>), 2 x 25.9 (2 x CH<sub>2</sub>).

**HR-ESI-MS** ( $m/z$ ): 463.1922 [M+H]<sup>+</sup> (calc. 463.1911 [M+H]<sup>+</sup>, C<sub>18</sub>H<sub>26</sub>F<sub>3</sub>N<sub>6</sub>O<sub>5</sub><sup>+</sup>), deviation: 2.37 ppm.

### 1.2.13 Synthesis of N6-Trifluoroacetamidohexyladenosine 5'-Thiophosphate (**13**)



N6-Trifluoroacetamidohexyladenosine (200 mg, 432  $\mu$ mol, 1.0 eq.) was dissolved in dry pyridine (6 mL) and cooled to 0 °C. PSCl<sub>3</sub> (80.5 mg, 47.9  $\mu$ L, 475  $\mu$ mol, 1.1 eq.) was added and the reaction mixture was stirred at 0 °C for 2 h. The reaction was quenched by addition of TEAB buffer (0.2 M, pH = 7, 15 mL) and stirring at rt for 30 min. The solvents were evaporated and the residue was purified by anion-exchange chromatography and RP-HPLC. The pure product **13** (194  $\mu$ mol, 45%) was obtained as a white solid.

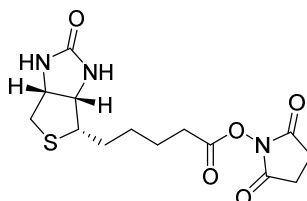
**<sup>1</sup>H NMR** (400 MHz, MeOD):  $\delta$  (ppm) = 8.65 (s, 1H, H-8), 8.25 (s, 1H, H-2), 6.11 (d,  $J$  = 6.0 Hz, 1H, H-1'), 4.72 (t,  $J$  = 5.5 Hz, 1H, H-2'), 4.47 (dd,  $J$  = 5.0, 3.1 Hz, 1H, H-3'), 4.28–4.23 (m, 1H, H-4'), 4.21–4.09 (m, 2H, H-5'<sub>a</sub>, H-5'<sub>b</sub>), 3.61 (s<sub>br</sub>, 2H, C6-NH<sub>2</sub>-CH<sub>2</sub>), 3.32–3.26 (m, 2H, CH<sub>2</sub>-linker), 1.71 (p,  $J$  = 7.3 Hz, 2H, CH<sub>2</sub>-linker), 1.60 (p,  $J$  = 7.3 Hz, 2H, CH<sub>2</sub>-linker), 1.54–1.38 (m, 4H, 2 x CH<sub>2</sub>-linker).

**<sup>31</sup>P NMR** (160 MHz, MeOD):  $\delta$  (ppm) = 49.2 (s, 1P, P<sub>S</sub>).

**<sup>19</sup>F NMR** (376 MHz, MeOD):  $\delta$  (ppm) = -77.37 (s, 1F, CF<sub>3</sub>).

**HR-ESI-MS** ( $m/z$ ): 557.1213  $[M-H]^-$  (calc. 557.1190  $[M-H]^-$ ,  $C_{18}H_{25}F_3N_6O_7PS^-$ ), deviation: 4.13 ppm.

#### 1.2.14 Synthesis of Biotin-NHS ester

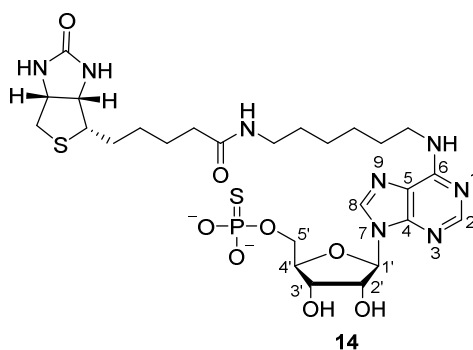


A solution of D-biotin (3.00 g, 12.3 mmol, 1.0 eq.), EDC · HCl (1.2 eq.) and *N*-hydroxysuccinimide (1.1 eq.) in DMF (100 mL) was stirred at rt overnight. The reaction mixture was concentrated and the residue was filtered and washed with EtOH/AcOH/H<sub>2</sub>O (95:1:4, 2 x 50 mL). The obtained product was dried *in vacuo* and used for the next reaction without further purification (3.76 g, 11.0 mmol, 90%).

**<sup>1</sup>H NMR** (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  (ppm) = 6.43 (s, 1H), 6.37 (s, 1H), 4.32–4.29 (m, 1H), 4.16–4.13 (m, 1H), 3.11 (ddd,  $J$  = 8.3, 6.4, 4.5 Hz, 1H), 2.84 (d,  $J$  = 5.1 Hz, 1H), 2.82 (d,  $J$  = 5.1 Hz, 1H), 2.67 (dd,  $J$  = 5.1, 4.5 Hz, 2H), 2.58 (d,  $J$  = 12.4 Hz, 1H), 1.69–1.60 (m, 3H), 1.54–1.46 (m, 1H), 1.45–1.37 (m, 2H).

**HR-ESI-MS** ( $m/z$ ): 342.1119  $[M+H]^+$  (calc. 342.1118  $[M+H]^+$ ,  $C_{14}H_{20}N_3O_5S^+$ ), deviation: 0.29 ppm.

#### 1.2.15 Synthesis of *N*<sup>6</sup>-Biotinylohexyladenosine 5'-Thiophosphate (14)



*N*<sup>6</sup>-Trifluoroacetamidohexyladenosine 5'-thiophosphate (145  $\mu$ mol, 1.0 eq.) was dissolved in aqueous NaOH solution (0.1 M, 10 mL) and stirred at rt for 4.5 h. NaHCO<sub>3</sub> buffer (1 M, 2 mL, pH 8.7) and biotin-NHS ester (59.4 mg, 174  $\mu$ mol, 1.2 eq.) in DMF (1 mL) were added, the pH was adjusted to 8.7 and the reaction mixture was stirred at rt overnight. The solvents were evaporated and purification by RP-HPLC yielded the product **14** (113  $\mu$ mol, 78%) as a white solid.

**HR-ESI-MS** ( $m/z$ ): 687.2141 (calc. 687.2143  $[M-H]^-$ ,  $C_{26}H_{40}N_8O_8PS_2^-$ ), deviation: 0.29 ppm.

**<sup>1</sup>H NMR** (400 MHz, D<sub>2</sub>O): δ (ppm) = 8.47 (s, 1H, H-8), 8.11–8.10 (m, 1H, H-2), 8.04 (d, *J* = 6.9 Hz, 1H, H-2), 6.02 (dd, *J* = 5.4, 2.1 Hz, 1H, H-1'), 5.85 (dd, *J* = 6.1, 4.6 Hz, 1H, H-1'), 4.96 (t, *J* = 6.3 Hz, 1H, H-2'), 4.69–4.62 (m, 1H, H-2'), 4.57–4.50 (m, 2H, 2 x H-3'), 4.48–4.43 (m, 1H, 84

H-4'), 4.41–4.36 (m, 1H, H-4'), 4.34–4.23 (m, 4H, 4 x H-5'), 4.22–4.14 (m, 2H, CO-CH<sub>2</sub>), 3.50–3.35 (m, 2H, C6-NH-CH<sub>2</sub>), 3.16–2.96 (m, 2H, CH<sub>2</sub>-linker), 2.85–2.77 (m, 1H, S-CH), 2.63 (d,  $J = 12.8$  Hz, S-CH-CH), 2.50 (dt,  $J = 20.6, 3.8$  Hz, 2H, P-CH<sub>2</sub>-P), 2.20 (t,  $J = 6.9$  Hz, 2H, S-CH<sub>2</sub>), 1.68–1.33 (m, 15H, 7 x CH<sub>2</sub>-linker, S-CH<sub>2</sub>-CH).

<sup>31</sup>P NMR (160 MHz, D<sub>2</sub>O):  $\delta$  (ppm) = 42.58 (dd,  $J = 53.3, 31.6$  Hz, P<sub>S</sub>), 17.17 (d,  $J = 8.3$  Hz, P <sub>$\alpha$</sub> ), 7.33 (dt,  $J = 31.6, 8.3$  Hz, P <sub>$\beta$</sub> ).

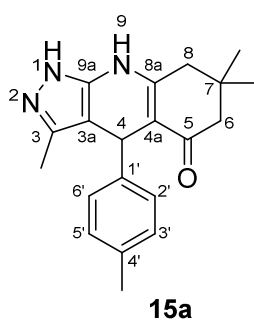
HR-ESI-MS ( $m/z$ ): 1135.2564 (calc. 1135.2552 [M-H]<sup>-</sup>, C<sub>37</sub>H<sub>54</sub>N<sub>16</sub>O<sub>16</sub>P<sub>3</sub>S<sub>2</sub><sup>-</sup>), deviation: 1.06 ppm.

### 1.3 Synthesis of Fhit Inhibitors

#### 1.3.1 General Synthesis of Cluster I Derivatives 15a–23a<sup>252-253</sup>

Dimedone (140 mg, 1.00 mmol, 1.0 eq.), 3-amino-5-methylpyrazole (97.0 mg, 1.00 mmol, 1.0 eq.), the respective aldehyde (1.00 mmol, 1.0 eq.) and triethylamine (121 mg, 166  $\mu$ L, 1.20 mmol, 1.2 eq.) were mixed in water (3.0 mL) and heated in a microwave reactor to 170 °C for 15 min. After cooling to room temperature an EtOH/H<sub>2</sub>O mixture (1:1, 3 mL) was added and the reaction stirred for 5 min. The respective product was gained by filtration, washed with EtOH/H<sub>2</sub>O (1:1, 15 mL) and dried *in vacuo*.

##### 1.3.1.1 3,7,7-Trimethyl-4-(4-ethylphenyl)-1,4,6,7,8,9-hexahydro-1H-pyrazolo[3,4-*b*]-quinoline-1-one (15a)



TLC:  $R_f = 0.32$  (DCM/MeOH 10:1).

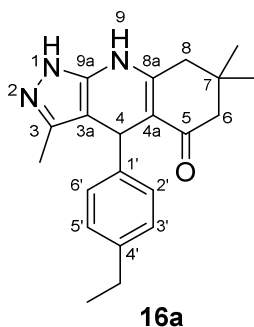
<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 11.68 (s, 1H, N1-H), 9.62 (s, 1H, N9-H), 6.98 (q,  $J = 8.0$  Hz, 4H, H-Ar), 4.87 (s, 1H, H-4), 2.41 (q,  $J = 16.0$  Hz, 2H, H-8), 2.19 (s, 3H, C4'-CH<sub>3</sub>), 2.10 (d,  $J = 16.0$  Hz, 1H, H-6<sub>a</sub>), 1.93 (d,  $J = 16.0$  Hz, 1H, H-6<sub>b</sub>), 1.89 (s, 3H, C3-CH<sub>3</sub>), 1.00 (s, 3H, C7-CH<sub>3</sub>), 0.93 (s, 3H, C7-CH<sub>3</sub>).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 192.7 (C-5), 152.5 (C-4a), 146.3 (C-3), 145.5 (C-1'), 134.7 (C-9a), 133.8 (C-4'), 128.2 (C-3', C-5'), 127.0 (C-2', C-6'), 107.1 (C-8a), 104.0 (C-3a), 50.5

(C-6), 41.0 (C-8), 34.6 (C-4), 31.8 (C-7), 28.9 (C7-CH<sub>3</sub>), 26.8 (C7-CH<sub>3</sub>), 20.5 (C4'-CH<sub>3</sub>), 9.3 (C3-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 322.1915 [M+H]<sup>+</sup> (calc. 322.1914 [M+H]<sup>+</sup>), deviation: 0.31 ppm.

### 1.3.1.2 3,7,7-Trimethyl-4-(4-ethylphenyl)-1,4,6,7,8,9-hexahydro-1H-pyrazolo[3,4-*b*]-quinoline-1-one (16a)



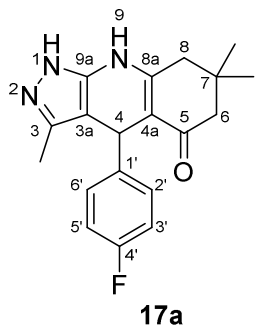
TLC:  $R_f$  = 0.44 (DMC/MeOH 10:1).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 11.68 (s, 1H, N1-H), 9.61 (s, 1H, N9-H), 7.05–6.97 (m, 4H, H-Ar), 4.88 (s, 1H, H-4), 2.52–2.47 (m, 2H, H-7'), 2.42 (q,  $J$  = 16.0 Hz, 2H, H-8<sub>a,b</sub>), 2.10 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>b</sub>), 1.94 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>a</sub>), 1.90 (s, 3H, C3-CH<sub>3</sub>), 1.12 (t,  $J$  = 8.0 Hz, 3H, H-8'), 1.00 (s, 3H, C7-CH<sub>3</sub>), 0.95 (s, 3H, C7-CH<sub>3</sub>).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 192.8 (C-5), 152.7 (C-4a), 146.3 (C-9a), 145.8 (C-1'), 140.2 (C-4'), 134.7 (C-3), 127.0 (C-2', C-3', C-5', C-6'), 107.1 (C-8a), 104.1 (C-3a), 50.5 (C-6), 41.0 (C-8), 34.6 (C-4), 31.9 (C-7), 28.8 (C7-CH<sub>3</sub>), 27.7 (C-7'), 26.9 (C7-CH<sub>3</sub>), 15.5 (C-8'), 9.4 (C3-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 336.2066 [M+H]<sup>+</sup> (calc. 336.2070 [M+H]<sup>+</sup>), deviation: 1.19 ppm.

### 1.3.1.3 3,7,7-Trimethyl-4-(4-fluorophenyl)-1,4,6,7,8,9-hexahydro-1H-pyrazolo[3,4-*b*]-quinoline-1-one (17a)



TLC:  $R_f$  = 0.22 (DCM/MeOH 10:1)

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 11.73 (s, 1H, N1-H), 9.68 (s, 1H, N9-H), 7.17–7.10 (m, 2H, H-3', H-5'), 7.01–6.94 (m, 2H, H-2', H-6'), 4.94 (s, 1H, H-4), 2.42 (q,  $J$  = 16.0 Hz, 2H, H-

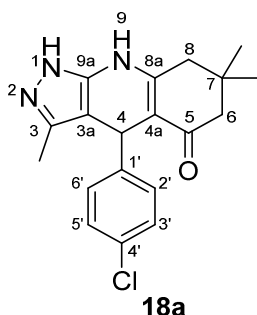
8<sub>a,b</sub>), 2.11 (d,  $J = 16.0$  Hz, 1H, H-6<sub>b</sub>), 1.95 (d,  $J = 16.0$  Hz, 1H, H-6<sub>a</sub>), 1.88 (s, 3H, C3-CH<sub>3</sub>), 1.00 (s, 3H, C7-CH<sub>3</sub>).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 192.7 (C-5), 152.7 (C-4a), 146.2 (C-9a), 144.6 (C-4'), 134.9 (C-3), 128.8 (C-2'), 128.7 (C-6'), 114.3 (C-3'), 114.1 (C-5'), 106.9 (C-8a), 103.7 (C-3a), 50.4 (C-6), 41.0 (C-8), 34.4 (C-4), 31.8 (C-7), 28.7 (C7-CH<sub>3</sub>), 26.9 (C7-CH<sub>3</sub>), 9.3 (C3-CH<sub>3</sub>).

<sup>19</sup>F NMR (377 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = -118.4 (s, F-Ar).

HR-ESI-MS ( $m/z$ ): 326.1646 [M+H]<sup>+</sup> (calc. 326.1663 [M+H]<sup>+</sup>), deviation: 5.21 ppm.

#### 1.3.1.4 3,7,7-Trimethyl-4-(4-chlorophenyl)-1,4,6,7,8,9-hexahydro-1H-pyrazolo[3,4-*b*]-quinoline-1-one (18a)

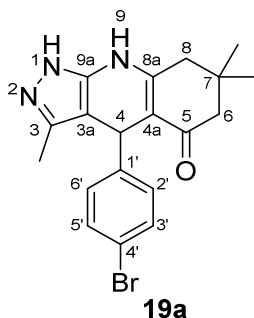


TLC:  $R_f = 0.37$  (DCM/MeOH 10:1).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 11.76 (s, 1H, N1-H), 9.72 (s, 1H, N9-H), 7.23–7.21 (m, 2H, H-3', H-5'), 7.14–7.12 (m, 2H, H-2', H-6'), 4.93 (s, 1H, H-4), 2.42 (q,  $J = 16.0$  Hz, 2H, H-8<sub>a,b</sub>), 2.11 (d,  $J = 16.0$  Hz, 1H, H-6<sub>b</sub>), 1.94 (d,  $J = 16.0$  Hz, 1H, H-6<sub>a</sub>), 1.88 (s, 3H, C3-CH<sub>3</sub>), 1.00 (s, 3H, C7-CH<sub>3</sub>), 0.92 (C7-CH<sub>3</sub>).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 192.3 (C-5), 152.4 (C-4a), 146.8 (C-4'), 145.7 (C-3), 134.5 (C-9a), 129.1 (C-1'), 128.5 (C-2', C-6'), 127.1 (C-3', C-5'), 106.1 (C-8a), 102.9 (C-3a), 49.9 (C-6), 40.5 (C-8), 34.1 (C-4), 31.4 (C-7), 28.3 (C7-CH<sub>3</sub>), 26.4 (C7-CH<sub>3</sub>), 8.8 (C3-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 342.1367 [M+H]<sup>+</sup> (calc. 342.1368 [M+H]<sup>+</sup>), deviation: 0.29 ppm.

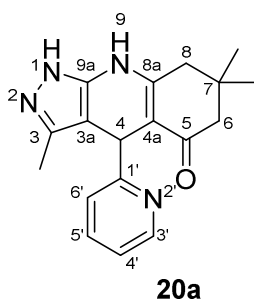
**1.3.1.5 3,7,7-Trimethyl-4-(4-bromophenyl)-1,4,6,7,8,9-hexahydro-1H-pyrazolo[3,4-*b*]-quinoline-1-one (19a)**

TLC:  $R_f$  = 0.51 (DCM/MeOH 10:1).

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  [ppm] = 11.76 (s, 1H, N1-H), 9.72 (s, 1H, N9-H), 7.38–7.34 (m, 2H, H-3', H-5'), 7.10–7.05 (m, 2H, H-2', H-6'), 4.92 (s, 1H, H-4), 2.42 (q,  $J$  = 16.0 Hz, 2H, H-8<sub>a,b</sub>), 2.11 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>b</sub>), 1.94 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>a</sub>), 1.88 (s, 3H, C3-CH<sub>3</sub>), 1.00 (s, 3H, C7-CH<sub>3</sub>), 0.92 (s, 3H, C7-CH<sub>3</sub>).

$^{13}\text{C}$  NMR (101 MHz, DMSO- $d_6$ ):  $\delta$  [ppm] = 192.7 (C-5), 152.8 (C-4a), 147.7 (C-1'), 146.2 (C-9a), 135.0 (C-3), 130.5 (C-2', C-6'), 129.4 (C-3', C-5'), 118.0 (C-4'), 106.5 (C-8a), 103.3 (C-3a), 50.4 (C-6), 41.0 (C-8), 34.7 (C-4), 31.9 (C-7), 28.7 (C7-CH<sub>3</sub>), 26.9 (C7-CH<sub>3</sub>), 9.3 (C3-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 386.0844 [ $\text{M}+\text{H}$ ]<sup>+</sup> (calc. 386.0863 [ $\text{M}+\text{H}$ ]<sup>+</sup>), deviation: 4.92 ppm.

**1.3.1.6 3,7,7-Trimethyl-4-(2-pyridinyl)-1,4,6,7,8,9-hexahydro-1H-pyrazolo[3,4-*b*]-quinoline-1-one (20a)**

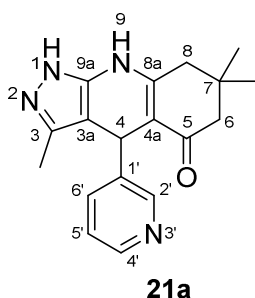
TLC:  $R_f$  = 0.34 (DCM/MeOH 10:1).

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  [ppm] = 11.68 (s, 1H, N1-H), 9.65 (s, 1H, N9-H), 8.37–8.32 (m, 1H, H-3'), 7.59 (td,  $J$  = 7.6, 1.9 Hz, 1H, H-5'), 7.13 (dt,  $J$  = 7.6, 1.0 Hz, 1H, H-6'), 7.05 (ddd,  $J$  = 7.6 Hz,  $J$  = 4.8, 1.0 Hz, 1H, H-4'), 5.08 (s, 1H, H-4), 2.45 (dd,  $J$  = 20.4, 16.8 Hz, 2H, H-8), 2.12 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>a</sub>), 1.98 (s, 3H, C3-CH<sub>3</sub>), 1.97 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>b</sub>), 1.01 (s, 6H, C7-(CH<sub>3</sub>)<sub>2</sub>).

$^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  [ppm] = 192.5 (C-5), 165.9 (C-1'), 153.7 (C-4a), 148.3 (C-3'), 146.3 (C-9a), 136.0 (C-5'), 135.3 (C-3), 121.1 (C-6'), 120.6 (C-4'), 105.6 (C-8a), 102.7 (C-3a), 50.4 (C-6), 41.1 (C-8), 38.1 (C-4), 32.0 (C-7), 28.7 (C7-CH<sub>3</sub>), 27.2 (C7-CH<sub>3</sub>), 9.6 (C3-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 309.1699  $[\text{M}+\text{H}]^+$  (calc. 309.1710  $[\text{M}+\text{H}]^+$ ), deviation: 3.56 ppm.

### 1.3.1.7 3,7,7-Trimethyl-4-(3-pyridinyl)-1,4,6,7,8,9-hexahydro-1H-pyrazolo[3,4-*b*]-quinoline-1-one (21a)



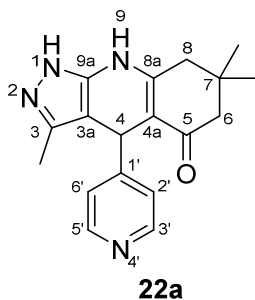
TLC:  $R_f$  = 0.32 (DCM/MeOH 10:1).

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  [ppm] = 11.79 (s, 1H, N1-H), 9.76 (s, 1H, N9-H), 8.38 (d,  $J$  = 1.8 Hz, 1H, H-2'), 8.25 (dd,  $J$  = 4.8, 1.8 Hz, 1H, H-4'), 7.43 (dt,  $J$  = 7.6, 1.8 Hz, 1H, H-6'), 7.20 (dd,  $J$  = 7.6, 4.8 Hz, 1H, H-5'), 4.96 (s, 1H, H-4), 2.44 (dd,  $J$  = 16.0, 6.6 Hz, 2H, H-8), 2.12 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>a</sub>), 1.95 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>b</sub>), 1.88 (s, 3H, C3-CH<sub>3</sub>), 1.00 (s, 3H, C7-CH<sub>3</sub>), 0.92 (s, 3H, C7-CH<sub>3</sub>).

$^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ ):  $\delta$  [ppm] = 192.7 (C-5), 153.1 (C-4a), 148.6 (C-2'), 146.4 (C-9a), 146.2 (C-4'), 143.3 (C-1'), 135.1 (C-3), 134.5 (C-6'), 123.2 (C-5'), 106.1 (C-8a), 103.0 (C-3a), 50.3 (C-6), 41.0 (C-8), 32.8 (C-4), 31.9 (C-7), 28.7 (C7-CH<sub>3</sub>), 26.9 (C7-CH<sub>3</sub>), 9.2 (C3-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 309.1699  $[\text{M}+\text{H}]^+$  (calc. 309.1710  $[\text{M}+\text{H}]^+$ ), deviation: 3.56 ppm.

### 1.3.1.8 3,7,7-Trimethyl-4-(4-pyridinyl)-1,4,6,7,8,9-hexahydro-1H-pyrazolo[3,4-*b*]-quinoline-1-one (22a)



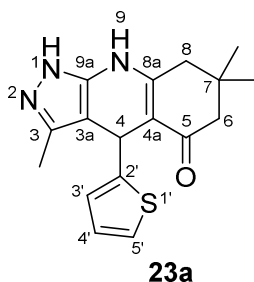
TLC:  $R_f = 0.21$  (DCM/MeOH 10:1).

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  [ppm] = 11.81 (s, 1H, N1-H), 9.78 (s, 1H, N9-H), 8.37–8.34 (m, 2H, H-2', H-6'), 7.13–7.08 (m, 2H, H-3', H-5'), 4.94 (s, 1H, H-4), 2.45 (dd,  $J = 16.0, 6.4$  Hz, 2H, H-8<sub>a,b</sub>), 2.13 (d,  $J = 16.0$  Hz, 1H, H-6<sub>a</sub>), 1.97 (d,  $J = 16.0$  Hz, 1H, H-6<sub>b</sub>), 1.90 (s, 3H, C3-CH<sub>3</sub>), 1.00 (s, 3H, C7-CH<sub>3</sub>), 0.94 (s, 3H, C7-CH<sub>3</sub>).

$^{13}\text{C}$  NMR (101 MHz, DMSO- $d_6$ ):  $\delta$  [ppm] = 192.8 (C-5), 156.2 (C-1'), 153.4 (C-4a), 149.1 (C-2', C-6'), 146.1 (C-9a), 135.3 (C-3), 122.6 (C-3', C-5'), 105.6 (C-8a), 102.3 (C-3a), 50.3 (C-6), 41.0 (C-8), 34.9 (C-4), 31.9 (C-7), 28.7 (C7-CH<sub>3</sub>), 26.9 (C7-CH<sub>3</sub>), 9.3 (C3-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 309.1700 [M+H]<sup>+</sup> (calc. 309.1710 [M+H]<sup>+</sup>), deviation: 3.23 ppm.

### 1.3.1.9 3,7,7-Trimethyl-4-(thiophen-2-yl)-1,4,6,7,8,9-hexahydro-1H-pyrazolo[3,4-*b*]-quinoline-1-one (23a)



TLC:  $R_f = 0.45$  (DCM/MeOH 10:1).

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta$  [ppm] = 11.83 (s, 1H, N1-H), 9.73 (s, 1H, N9-H), 7.13 (dd,  $J = 5.1, 1.2$  Hz, 1H, H-5'), 6.79 (dd,  $J = 5.1, 3.4$  Hz, 1H, H-4'), 6.74 (dt,  $J = 3.4, 1.2$  Hz, 1H, H-3'), 5.31 (s, 1H, H-4), 2.39 (q,  $J = 16.0$  Hz, 2H, H-8<sub>a,b</sub>), 2.15 (d,  $J = 16.0$  Hz, 1H, H-6<sub>b</sub>), 2.05 (s, 3H, C3-CH<sub>3</sub>), 2.03 (d,  $J = 16.0$  Hz, 1H, H-6<sub>a</sub>), 1.00 (s, 3H, C7-CH<sub>3</sub>), 0.98 (s, 3H, C7-CH<sub>3</sub>).

$^{13}\text{C}$  NMR (101 MHz, DMSO- $d_6$ ):  $\delta$  [ppm] = 192.8 (C-5), 153.1 (C-2), 152.7 (C-4a), 146.2 (C-3), 135.1 (C-9a), 126.1 (C-5'), 122.9 (C-4'), 122.3 (C-3'), 106.8 (C-8a), 103.2 (C-3a), 50.4 (C-6), 40.9 (C-8), 31.8 (C-7), 29.5 (C-4), 28.9 (C7-CH<sub>3</sub>), 26.8 (C7-CH<sub>3</sub>), 9.4 (C3-CH<sub>3</sub>).

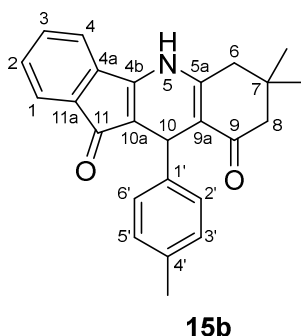
HR-ESI-MS ( $m/z$ ): 314.1317 [M+H]<sup>+</sup> (calc. 314.1322 [M+H]<sup>+</sup>), deviation: 1.59 ppm.

### 1.3.2 General Synthesis of Cluster II Derivatives 15b–23b<sup>254-255</sup>

1,3-Indanedione (147 mg, 1.00 mmol, 1.0 eq.), 5,5-dimethyl-3-aminocyclohex-2-enone (139 mg, 1.00 mmol, 1.0 eq.), the respective aldehyde (1.00 mmol, 1.0 eq.) and *p*-TsOH monohydrate

(190 mg, 1.00 mmol, 1.0 eq.) were mixed in water (1 mL) and heated in a microwave reactor to 150 °C for 10 min. The reaction was cooled to room temperature, quenched with 10% NaOH (0.4 mL) and stirred for 15 min. The resulting suspension was diluted with EtOH (3 mL), the residue was filtered and washed with EtOH (15 mL). Column chromatography on silica gel (DCM/MeOH, 100:1 → 10:1), subsequent purification via MPLC and drying *in vacuo* gave the respective product.

### 1.3.2.1 10-(4-Methylphenyl)-7,7-dimethyl-6,7,8,10-tetrahydro-1*H*-indeno[1,2-*b*]quinolone-9,11-dione (15b)



**TLC:**  $R_f$  = 0.70 (RP silica gel, MeCN/H<sub>2</sub>O 10:1).

**<sup>1</sup>H NMR** (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 10.38 (s, 1H, *N*-H), 7.55 (d,  $J$  = 7.3 Hz, 1H, H-4), 7.43 (td,  $J$  = 7.3, 1.1 Hz, 1H, H-2), 7.32 (td,  $J$  = 7.3, 1.1 Hz, 1H, H-3), 7.23 (d,  $J$  = 7.3 Hz, 1H, H-1), 7.08 (d,  $J$  = 8.0 Hz, 2H, H-2', H-6'), 6.99 (d,  $J$  = 8.0 Hz, 2H, H-3', H-5'), 4.66 (s, 1H, H-10), 2.63 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>a</sub>), 2.56 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>b</sub>), 2.24 (d,  $J$  = 16.0 Hz, 1H, H-8<sub>a</sub>), 2.19 (s, 3H, C4'-CH<sub>3</sub>), 2.08 (d,  $J$  = 16.0 Hz, 1H, H-8<sub>b</sub>), 1.06 (s, 3H, C7-CH<sub>3</sub>), 0.98 (s, 3H, C7-CH<sub>3</sub>).

**<sup>13</sup>C NMR** (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 194.2 (C-9), 190.5 (C-11), 153.0 (C-10a), 149.3 (C-9a), 142.7 (C-1'), 135.7 (C-11a), 134.2 (C-4'), 132.5 (C-4a), 131.5 (C-2), 129.5 (C-3), 127.9 (C-3', C-5'), 126.9 (C-2', C-6'), 119.9 (C-1), 118.5 (C-4), 112.8 (C-5a), 109.7 (C-4b), 49.8 (C-8), 32.6 (C-10), 31.5 (C-7), 28.1 (C7-CH<sub>3</sub>), 26.3 (C7-CH<sub>3</sub>), 20.0 (C4'-CH<sub>3</sub>).

**HR-ESI-MS** ( $m/z$ ): 370.1785 [M+H]<sup>+</sup> (calc. 370.1802 [M+H]<sup>+</sup>), deviation: 4.59 ppm.

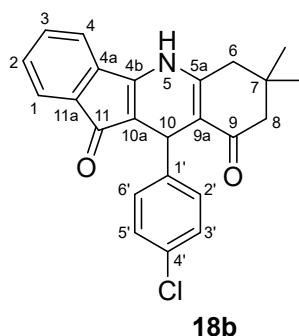


7.07–6.98 (m, 2H, H-3', H-5'), 4.73 (s, 1H, H-10), 2.63 (d,  $J = 16.0$  Hz, 1H, H-6<sub>a</sub>), 2.58 (d,  $J = 16.0$  Hz, 1H, H-6<sub>b</sub>), 2.24 (d,  $J = 16.0$  Hz, 1H, H-8<sub>b</sub>), 2.10 (d,  $J = 16.0$  Hz, 1H, H-8<sub>a</sub>), 1.06 (s, 3H, C7-CH<sub>3</sub>), 0.98 (s, 3H, C7-CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 194.8 (C-9), 191.0 (C-11), 159.7 (C-4'), 153.7 (C-10a), 150.1 (C-9a), 142.2 (C-1'), 136.2 (C-11a), 132.9 (C-4a), 131.9 (C-2), 130.1 (C-3), 129.3 (C-2, C-6'), 120.6 (C-1), 119.2 (C-4), 114.6 (C-3', C-5'), 113.1 (C-5a), 109.8 (C-4b), 50.3 (C-8), 40.1 (C-6), 33.0 (C-10), 32.1 (C-7), 28.6 (C7-CH<sub>3</sub>), 26.9 (C7-CH<sub>3</sub>).

<sup>19</sup>F NMR (377 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = -117.3 (s, F-Ar).

HR-ESI-MS ( $m/z$ ): 374.1546 [M+H]<sup>+</sup> (calc. 374.1551 [M+H]<sup>+</sup>), deviation: 1.34 ppm.

#### 1.3.2.4 10-(4-Chlorophenyl)-7,7-dimethyl-6,7,8,10-tetrahydro-1H-indeno[1,2-*b*]quinolone-9,11-dione (18b)

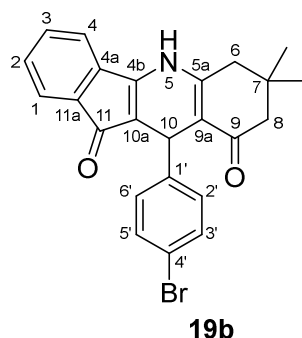


TLC:  $R_f = 0.73$  (RP silica gel, MeCN/H<sub>2</sub>O 10:1).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 10.38 (s, 1H, N-H), 7.57 (d,  $J = 7.1$  Hz, 1H, H-4), 7.44 (t,  $J = 7.3$  Hz, 1H, H-2), 7.33 (t,  $J = 7.3$  Hz, 1H, H-3), 7.29–7.18 (m, 5H, H-1, H-2', H-3', H-5', H-6'), 4.72 (s, 1H, H-10), 2.63 (d,  $J = 17.3$  Hz, 1H, H-6<sub>a</sub>), 2.58 (d,  $J = 17.3$  Hz, 1H, H-6<sub>b</sub>), 2.24 (d,  $J = 16.1$  Hz, 1H, H-8<sub>b</sub>), 2.10 (d,  $J = 16.0$  Hz, 1H, H-8<sub>a</sub>), 1.06 (s, 3H, C7-CH<sub>3</sub>), 0.98 (s, 3H, C7-CH<sub>3</sub>). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 195.3 (C-9), 191.5 (C-11), 154.3 (C-10a), 150.8 (C-9a), 145.5 (C-1'), 136.6 (C-11a), 133.1 (C-4a), 132.4 (C-2), 130.9 (C-3), 130.7 (C-4'), 129.9 (C-3', C-5'), 128.4 (C-2', C-6'), 121.1 (C-1), 119.8 (C-4), 113.4 (C-5a), 110.0 (C-4b), 50.8 (C-8), 40.7 (C-6), 33.8 (C-10), 32.6 (C-7), 29.1 (C7-CH<sub>3</sub>), 27.4 (C7-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 412.1059 [M+Na]<sup>+</sup> (calc. 412.1075 [M+Na]<sup>+</sup>), deviation: 3.88 ppm.

### 1.3.2.5 10-(4-Bromophenyl)-7,7-dimethyl-6,7,8,10-tetrahydro-1H-indeno[1,2-*b*]quinolone-9,11-dione (19b)



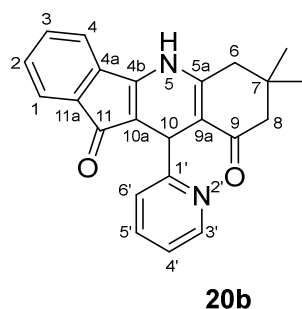
TLC:  $R_f$  = 0.69 (RP silica gel, MeCN/H<sub>2</sub>O 10:1).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 10.45 (s, 1H, *N*-H), 7.57 (d,  $J$  = 7.3 Hz, 1H, H-4), 7.45 (td,  $J$  = 7.3, 1.1 Hz, 1H, H-2), 7.42–7.37 (m, 2H, H-2', H-6'), 7.34 (td,  $J$  = 7.3, 1.1 Hz, 1H, H-3), 7.25 (d,  $J$  = 7.3 Hz, 1H, H-1), 7.19–7.13 (m, 2H, H-3', H-5'), 4.70 (s, 1H, H-10), 2.63 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>a</sub>), 2.58 (d,  $J$  = 16.0 Hz, 1H, H-6<sub>b</sub>), 2.24 (d,  $J$  = 16.0 Hz, 1H, H-8<sub>b</sub>), 2.10 (d,  $J$  = 16.0 Hz, 1H, H-8<sub>a</sub>), 1.06 (s, 3H, C7-CH<sub>3</sub>), 0.98 (s, 3H, C7-CH<sub>3</sub>).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 194.8 (C-9), 191.0 (C-11), 153.8 (C-10a), 150.3 (C-9a), 145.4 (C-1'), 136.1 (C-11a), 132.9 (C-4a), 131.9 (C-2), 130.8 (C-3', C-5'), 130.2 (C-2), 129.9 (C-2', C-6'), 120.6 (C-1), 119.3 (C-4), 118.9 (C-4'), 112.8 (C-5a), 109.5 (C-4b), 50.3 (C-8), 33.4 (C-10), 32.1 (C-7), 28.6 (C7-CH<sub>3</sub>), 26.9 (C7-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 436.0718 [M+H]<sup>+</sup> (calc. 436.0733 [M+H]<sup>+</sup>), deviation: 3.44 ppm.

### 1.3.2.6 10-(2-Pyridinyl)-7,7-dimethyl-6,7,8,10-tetrahydro-1H-indeno[1,2-*b*]quinolone-9,11-dione (20b)



TLC:  $R_f$  = 0.77 (RP silica gel, MeCN/H<sub>2</sub>O 10:1).

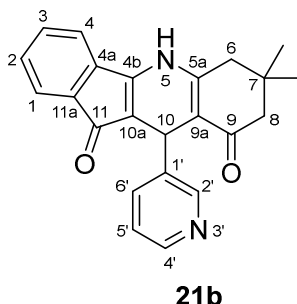
<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 10.34 (s, 1H, *N*-H), 8.34–8.30 (m, 1H, H-3'), 7.63 (td,  $J$  = 7.7, 1.4 Hz, 1H, H-5'), 7.54 (d,  $J$  = 7.2 Hz, 1H, H-4), 7.43 (td,  $J$  = 7.2, 1.0 Hz, 1H, H-2), 7.39 (d,  $J$  = 7.8 Hz, 1H, H-6'), 7.32 (td,  $J$  = 7.2, 1.0 Hz, 1H, H-3), 7.23 (d,  $J$  = 7.0 Hz, 1H, H-1), 7.08 (ddd,

$J = 7.7, 4.8, 1.4$  Hz, 1H, H-4'), 4.87 (s, 1H, H-10), 2.62–2.54 (m, 2H, H-6<sub>a,b</sub>), 2.25 (d,  $J = 16.0$  Hz, 1H, H-8<sub>a</sub>), 2.05 (d,  $J = 16.0$  Hz, 1H, H-8<sub>b</sub>), 1.07 (s, 3H, C7-CH<sub>3</sub>), 1.02 (s, 3H, C7-CH<sub>3</sub>).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 195.3 (C-9), 191.2 (C-11), 162.9 (C-1'), 154.6 (C-10a), 151.0 (C-3'), 150.9 (C-9a), 136.3 (C-11a), 133.2 (C-4a), 132.0 (C-2), 130.3 (C-3), 123.4 (C-6'), 121.8 (C-5'), 121.7 (C-4'), 120.7 (C-1), 119.3 (C-4), 112.6 (C-5a), 108.8 (C-4b), 50.4 (C-8), 40.3 (C-6), 36.3 (C-10), 32.4 (C-7), 28.9 (C7-CH<sub>3</sub>), 26.7 (C7-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 357.1581 [M+H]<sup>+</sup> (calc. 357.1598 [M+H]<sup>+</sup>), deviation: 4.76 ppm.

### 1.3.2.7 10-(3-Pyridinyl)-7,7-dimethyl-6,7,8,10-tetrahydro-1*H*-indeno[1,2-*b*]quinolone-9,11-dione (21b)



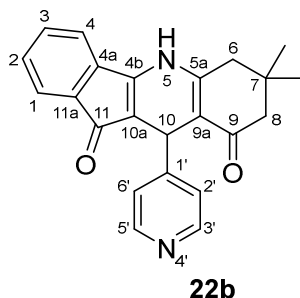
TLC:  $R_f = 0.71$  (RP silica gel, MeCN/H<sub>2</sub>O 10:1).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 10.49 (s, 1H, N-H), 8.45 (d,  $J = 1.9$  Hz, 1H, H-2'), 8.29 (dd,  $J = 4.8, 2.0$  Hz, 1H, H-4'), 7.61–7.54 (m, 2H, H-4, H-5'), 7.45 (td,  $J = 7.5, 1.1$  Hz, 1H, H-2), 7.34 (td,  $J = 7.5, 1.1$  Hz, 1H, H-3), 7.28–7.21 (m, 2H, H-1, H-6'), 4.75 (8s, 1H, H-10), 2.62 (s, 2H, H-6<sub>a,b</sub>), 2.25 (d,  $J = 16.0$  Hz, 1H, H-8<sub>a</sub>), 2.11 (d,  $J = 16.0$  Hz, 1H, H-8<sub>b</sub>), 1.07 (s, 3H, C7-CH<sub>3</sub>), 0.99 (s, 3H, C7-CH<sub>3</sub>).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 194.7 (C-9), 190.9 (C-11), 154.0 (C-10a), 150.6 (C-9a), 149.0 (C-2'), 147.0 (C-4'), 141.1 (C-1'), 136.0 (C-11a), 134.9 (C-5'), 132.8 (C-4a), 131.9 (C-2), 130.2 (C-3), 123.3 (C-6'), 120.6 (C-1), 119.3 (C-4), 112.3 (C-5a), 109.0 (C-4b), 50.2 (C-8), 40.1 (C-6), 32.1 (C-7), 31.7 (C-10), 28.5 (C7-CH<sub>3</sub>), 26.9 (C7-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 357.1589 [M+H]<sup>+</sup> (calc. 357.1598 [M+H]<sup>+</sup>), deviation: 2.52 ppm.

### 1.3.2.8 10-(4-Pyridinyl)-7,7-dimethyl-6,7,8,10-tetrahydro-1*H*-indeno[1,2-*b*]quinolone-9,11-dione (22b)



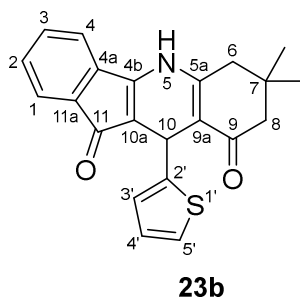
TLC:  $R_f$  = 0.51 (RP silica gel, MeCN/H<sub>2</sub>O 10:1).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 10.51 (s, 1H, *N*-H), 8.40 (dd,  $J$  = 4.8, 1.6 Hz, 2H, H-3', H-5'), 7.59 (d,  $J$  = 7.2 Hz, 1H, H-4), 7.46 (td,  $J$  = 7.7, 1.0 Hz, 1H, H-2), 7.35 (t,  $J$  = 7.4 Hz, 1H, H-3), 7.26 (d,  $J$  = 7.0 Hz, 1H, H-1), 7.21 (dd,  $J$  = 4.8, 1.6 Hz, 2H, H-2', H-6'), 4.73 (s, 1H, H-10), 2.63 (s, 2H, H-6), 2.26 (d,  $J$  = 16.1 Hz, 1H, H-8<sub>a</sub>), 2.13 (d,  $J$  = 16.1 Hz, 1H, H-8<sub>b</sub>), 1.07 (s, 3H, C7-CH<sub>3</sub>), 1.01 (s, 3H, C7-CH<sub>3</sub>).

<sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 194.7 (C-9), 190.8 (C-11), 154.2 (C-1'), 153.8 (C-10a), 150.9 (C-9a), 149.3 (C-3', C-5'), 136.0 (C-11a), 132.8 (C-4a), 131.9 (C-2), 130.3 (C-3), 122.9 (C-2', C-6'), 120.7 (C-1), 119.4 (C-4), 111.9 (C-5a), 108.5 (C-4b), 50.2 (C-8), 33.6 (C-10), 32.1 (C-7), 28.5 (C7-CH<sub>3</sub>), 27.0 (C7-CH<sub>3</sub>).

HR-ESI-MS ( $m/z$ ): 355.1441 [M+H, oxidized form]<sup>+</sup> (calc. 355.1441 [M+H, oxidized form]<sup>+</sup>), deviation: 0.00 ppm.

### 1.3.2.9 10-(Thiophen-2-yl)-7,7-dimethyl-6,7,8,10-tetrahydro-5*H*-indeno[1,2-*b*]quinolone-9,11-dione (23b)



TLC:  $R_f$  = 0.80 (RP silica gel, MeCN/H<sub>2</sub>O 10:1).

<sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 10.53 (s, 1H, *N*-H), 7.57 (d,  $J$  = 7.2 Hz, 1H, H-4), 7.45 (td,  $J$  = 7.3, 1.2 Hz, 1H, H-2), 7.36 (td,  $J$  = 7.3, 1.2 Hz, 1H, H-3), 7.33–7.29 (m, 1H, H-1), 7.19 (dd,  $J$  = 5.1, 1.3 Hz, 1H, H-5'), 6.83 (dd,  $J$  = 5.1, 3.5 Hz, 1H, H-4'), 6.79–6.76 (m, 1H, H-3'), 5.04 (s, 1H,

H-10), 2.62 (d,  $J = 16.0$  Hz, 1H, H-6<sub>a</sub>), 2.57 (d,  $J = 16.0$  Hz, 1H, H-6<sub>b</sub>), 2.29 (d,  $J = 16.1$  Hz, 1H, H-8<sub>a</sub>), 2.16 (d,  $J = 16.1$  Hz, 1H, H-8<sub>b</sub>), 1.07 (s, 3H, C7-CH<sub>3</sub>), 1.02 (s, 3H, C7-CH<sub>3</sub>).

**<sup>13</sup>C NMR** (101 MHz, DMSO-*d*<sub>6</sub>):  $\delta$  [ppm] = 194.8 (C-9), 190.9 (C-11), 153.9 (C-10a), 150.2 (C-2'), 150.0 (C-9a), 136.1 (C-11a), 132.9 (C-4a), 132.0 (C-2), 130.3 (C-3), 126.6 (C-5'), 123.7 (C-4'), 123.5 (C-3'), 120.8 (C-1), 119.3 (C-4), 113.0 (C-5a), 109.2 (C-4b), 50.3 (C-8), 40.1 (C-6), 32.0 (C-7), 28.8 (C7-CH<sub>3</sub>), 28.0 (C-10), 26.6 (C7-CH<sub>3</sub>).

**HR-ESI-MS** ( $m/z$ ): 384.1030 [M+Na]<sup>+</sup> (calc. 384.1029 [M+Na]<sup>+</sup>), deviation: 0.26 ppm.

## 2 Biochemical Experiments

### 2.1 List of General Buffers and Solutions

**Table 5:** List of general buffers and solutions and their composition.

| <b><i>Solution</i></b>                                         | <b><i>Composition</i></b>                                                                                                              |
|----------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|
| Coomassie Roti-Blue in methanol                                | 0.015% (w/v) Coomassie Roti-Blue ( <i>Roth</i> )<br>10% (v/v) acetic acid<br>50% (v/v) methanol<br>Stirred overnight and filtrated     |
| Coomassie staining solution                                    | 50% (v/v) Coomassie Roti-Blue in methanol<br>10% (v/v) acetic acid                                                                     |
| Destaining solution                                            | 50% (v/v) methanol<br>10% (v/v) acetic acid                                                                                            |
| LB medium                                                      | 2% (w/v) LB broth ( <i>Roth</i> )                                                                                                      |
| Rotiphorese® gel 30 (37.5:1) ( <i>Roth</i> )                   | 29.2% acrylamide<br>0.8% bisacrylamide                                                                                                 |
| PBS buffer (1x)                                                | 137 mM NaCl<br>2.7 mM KCl<br>12 mM NaH <sub>2</sub> PO <sub>4</sub> , pH 7.4                                                           |
| SDS-PAGE loading dye (6x)                                      | 225 mM Tris HCl (pH 6.8)<br>50% (v/v) glycerol<br>5% (w/v) SDS<br>0.05% (w/v) bromophenol blue<br>12.5% (v/v) $\beta$ -mercaptoethanol |
| SDS-PAGE running buffer (10x)                                  | 247.6 mM Tris<br>1.56 M glycine<br>34.7 mM SDS, pH 8.3                                                                                 |
| SDS-PAGE separating gel buffer<br>SDS-PAGE stacking gel buffer | 1.15 M Tris-HCl, pH 8.8<br>381 mM Tris-HCl, pH 6.8                                                                                     |
| TNE (20x)                                                      | 200 mM Tris<br>50 mM Na <sub>2</sub> EDTA · 2H <sub>2</sub> O<br>1 M NaCl                                                              |
| TNE-T                                                          | TNE<br>0.1% Triton X 100                                                                                                               |
| WB transfer buffer                                             | 20% MeOH<br>20% SDS-PAGE running buffer (1x)                                                                                           |

## 2.2 List of Utilized Machines

**Table 6:** List of utilized machines.

| <b><i>Machine</i></b>  | <b><i>Company</i></b>           | <b><i>Model</i></b>                         |
|------------------------|---------------------------------|---------------------------------------------|
| Cell counter           | <i>Invitrogen</i>               | Countess™ automated cell counter            |
| Centrifuges            | <i>Thermo Scientific</i>        | Sorvall LYNX 4000                           |
|                        | <i>Heraeus</i>                  | Multifuge 4 KR                              |
|                        | <i>Heraeus</i>                  | Primo R                                     |
|                        | <i>Eppendorf</i>                | Centrifuge 5810 R                           |
|                        | <i>Eppendorf</i>                | MiniSpin®                                   |
| Chromatography Systems | <i>Roth</i>                     | Rotilabo®-mini-centrifuge                   |
|                        | <i>GE Healthcare</i>            | ÄKTA pure                                   |
|                        | <i>Shimadzu</i>                 | Prominence LC-20AT (analytical)             |
|                        | <i>Zirbus</i>                   | VaCo 2-II-E                                 |
|                        | <i>Leybold-Heraeus</i>          | Lyovac GT2                                  |
| Freeze-dryers          | <i>Christ</i>                   | Beta 2-8 LDplus                             |
|                        | <i>Bio-Rad</i>                  | PowerPac™ basic                             |
|                        | <i>Bio-Rad</i>                  | PowerPac™ 3000                              |
|                        | <i>Bio-Rad</i>                  | PowerPac™ HC                                |
| Gel power supply       | <i>Bio-Rad</i>                  | PowerPac™ HC                                |
| Ice Machine            | <i>Scotsman</i>                 | AF103 Ice Flaker                            |
|                        | <i>Bio-Rad</i>                  | Molecular Imager® ChemiDoc™ XRS             |
| Imager                 | <i>GE Healthcare</i>            | Amersham Imager 600                         |
|                        | <i>Fujifilm</i>                 | LAS 3000                                    |
|                        | <i>Fujifilm</i>                 | FLA-5000                                    |
|                        | <i>GE Healthcare</i>            | Typhoon FLA-9500                            |
|                        | <i>GE Healthcare</i>            | Typhoon FLA-9500                            |
| Incubators             | <i>New Brunswick Scientific</i> | Innova®44                                   |
|                        | <i>Binder</i>                   | CB 160                                      |
| Microscope             | <i>Nikon</i>                    | ECLIPSE TS100                               |
| MilliQ                 | <i>Merck</i>                    | Biopak® Polisher                            |
| MS                     | <i>Thermo Fisher</i>            | LTQ Orbitrap                                |
| pH electrodes          | <i>Thermo Scientific</i>        | Orion Star™ A111                            |
|                        | <i>Mettler Toledo</i>           | SevenEasy™ pH                               |
| Photometer             | <i>Eppendorf</i>                | BioPhotometer 6131                          |
| Pipet Boy              | <i>PeqLab</i>                   | Nano Drop ND-1000                           |
|                        | <i>Hirschmann</i>               | pipetus®                                    |
| Plate reader           | <i>Perkin Elmer</i>             | 1420 Multilabel Counter Victor <sup>3</sup> |
|                        | <i>Tecan</i>                    | Infinite F500                               |
| Shaker                 | <i>Phoenix Instruments</i>      | RS-TR 5                                     |

|                     |                          |                            |
|---------------------|--------------------------|----------------------------|
|                     | <i>Heidolph</i>          | Reax 2                     |
|                     | <i>IKA</i>               | KS 260 basic               |
| SpeedVac            | <i>Thermo Scientific</i> | SPD121P, RVT400 vapor trap |
| Sonofier            | <i>Bandelin Sonopuls</i> | MS72 Sonotrode, HD2200     |
|                     | <i>Branson</i>           | Sonifier 250               |
| ThermoMixer         | <i>Eppendorf</i>         | ThermoMixer F2.0           |
| Tissue Culture Hood | <i>Heraeus</i>           | HERAsafe                   |
| UV lamp             | <i>UVP, LLC</i>          | Blak Ray® B-100AP          |
| Vortex              | <i>Heidolph</i>          | Reax control               |

## 2.3 General Methods Applied in Biochemical Experiments

### 2.3.1 SDS-PAGE Analysis

All solutions subjected to SDS-PAGE analysis were diluted with 1 x SDS loading dye and heated to 95 °C for 5 min. The separating gel was cast with a gradient of acrylamide percentage (10 to 12.5 to 15%, containing 2.6% bis-acrylamide), separating gel buffer (3.75 mL), ammonium peroxodisulfate (0.1% (w/v), APS) and *N,N,N',N'*-tetramethyl-ethylenediamine (15 µL, TEMED) in a total volume of 15 mL. The stacking gel was cast using acrylamide (5%, containing 2.6% bis-acrylamide), stacking gel buffer (940 mL), APS (0.1% (w/v)) and TEMED (4 µL) in a total volume of 4 mL. The proteins were separated at a current of 50 mA using 1 x SDS-PAGE running buffer. Fluorescent gels were read-out using a FLA-5000 imaging reader (*Fuji*), Coomassie-staining was detected with a Molecular Imager® ChemiDoc™ XRS system (*Bio-Rad*)

### 2.3.2 WB Analysis

SDS-PAGE gels were transferred to a Immobilon®-P transfer membrane (*Millipore*) with WB transfer buffer at 300 mA for 40 min under cooling and stirring. The membranes were washed with MQ for 5 min at rt and blocked with RotiBlock (1x, *Roth*) or BSA (5% (w/v)) in TNE-T for 1 h at rt. Afterwards, the membranes were washed with TNE-T (3 x 5–10 min) and treated with a primary antibody at 4 °C overnight. Washing with TNE-T (3 x 5–10 min) was followed by treatment with a secondary antibody for 1 h at rt. After washing with TNE-T (3 x 10 min) the membrane was read-out using Pierce™ ECL Western Blotting Substrate (*Thermo Fisher*) in a mixture of 1:1 in a LAS-3000 (*Fujifilm*).

### 2.3.3 Determination of Protein Concentration via Bradford Assay

A straight calibration line was recorded with BSA concentrations between 0.0 and 0.5 mg/mL in the respective buffer in a total volume of 20  $\mu$ L. The protein samples were applied in different dilutions with the respective buffer in the same volume. RotiQuant (5x, *Roth*) was diluted with MQ, filtered and 980  $\mu$ L were given to the BSA and protein samples and mixed. After incubated for 15 min at rt the results were obtained by read-out with the Bradford channel of a BioPhotometer 6131 (*Eppendorf*).

### 2.3.4 Expression and Purification of Fhit Protein

The human wild-type Fhit as 6 x his-tagged protein was transformed from a pPROEX-1 vector into BL21(DE3)-RIL *E. coli* cells for recombinant expression. The cells were grown overnight in LB medium containing Carbenicillin (100  $\mu$ g/mL) at 37 °C, diluted by a factor 100 and grown until OD<sub>600</sub> was 0.8. The expression was induced by addition of IPTG (1 mM) and Fhit was expressed for 3 h at 37 °C. The cells were collected by centrifugation at 4 °C (4400 rpm, 20 min) and the pellet was resuspended in buffer A (50 mM sodium phosphate, pH = 6.0), containing aprotinin (1  $\mu$ g/mL), leupeptin (1  $\mu$ g/mL) and Pefabloc® SC (1  $\mu$ g/mL). All following steps were performed at 4 °C. The cells were lysed by sonication (5 x cycle, 20%, 2 x 30 s) and the supernatant cleared by centrifugation (15000 rpm, 30 min). The supernatant was incubated with Ni-NTA beads for 2 h, which were pre-washed with buffer A (3 x). After incubation the supernatant was removed and the beads were washed with buffer A (2 x) and buffer A containing imidazole (100 mM). Bound protein was eluted from the beads with imidazole (200 mM) in buffer A and the fractions containing pure Fhit protein were identified by SDS-PAGE. The elution buffer of the pooled pure fractions was exchanged to buffer B (50 mM Tris, pH = 8.0, 1 mM EDTA) using a PD-10 column and the total protein concentration was determined using the Bradford assay. The protocol was adapted from previously published methods.<sup>236,258</sup>

### 2.3.5 Cell Culture and Preparation of Human Cell Lysates

HEK 293T cells were maintained in DMEM supplement with 10% fetal bovine serum and penicillin/streptomycin at 37 °C with 5% CO<sub>2</sub> in the headspace. Cells were grown to 90% confluence. To prepare cell lysates the medium was sucked off and the cells were washed with ice cold 1 x PBS (2 mL). All following steps were performed at 4 °C. The cells were harvested by scratching into 1 x PBS (2 x 1 mL) and collected by centrifugation (1000 g, 10 min). The supernatant was removed and the pellet stored at -20 °C until use. HEK 293T cells were

resuspended in lysate buffer (1 x PBS, 10 mM MgCl<sub>2</sub>, aprotinin (1 µg/mL), leupeptin (1 µg/mL), Pefabloc® SC (1 µg/mL)), disrupted by sonication (10 x cycle, 8 x) and the supernatant was cleared by centrifugation (14000 rpm, 30 min). The protein content of the lysate was determined using the Bradford assay.

### 2.3.6 *In-Gel Digestion*

The Coomassie-stained gels were washed with water for 15 min, the respective bands were cut in small pieces, placed in a fresh tube and incubated with water (50 µL) for 15 min. The supernatant was removed, a mixture of acetonitrile/water (3:2, 50 µL) was added and the gel pieces were incubated for 30 min at rt. After removing the supernatant, the gel pieces were mixed with NH<sub>4</sub>HCO<sub>3</sub> (25 mM, 50 µL) and incubated for 15 min, before the steps of incubation with acetonitrile/water (3:2, 50 µL) and NH<sub>4</sub>HCO<sub>3</sub> (25 mM, 50 µL) were repeated twice. The gel pieces were then incubated with NH<sub>4</sub>HCO<sub>3</sub> (20 mM, 50 µL) for 15 min, the supernatant removed and DTT (10 mM) in NH<sub>4</sub>HCO<sub>3</sub> (20 mM, 100 µL) was added and the mixture incubated at 37 °C for 1 h. The supernatant was removed, the gel pieces were cooled to rt and darkened with aluminium foil. Iodoacetamide (50 mM) in NH<sub>4</sub>HCO<sub>3</sub> (20 mM, 100 µL) was added, followed by incubation at rt for 60 min. The supernatant was removed and the gel pieces were washed with NH<sub>4</sub>HCO<sub>3</sub> (20 mM, 100 µL) for 15 min, NH<sub>4</sub>HCO<sub>3</sub> (20 mM)/acetonitrile (1:1, 100 µL) for 10 min and pure acetonitrile (100 µL) for 10 min. The shrunk white gel pieces were completely dried by freeze-drying. The gel pieces were placed on ice and incubated with Trypsin (12.5 µg/mL, *Promega*) in NH<sub>4</sub>HCO<sub>3</sub> (20 mM, 100 µL) for 45 min. The Trypsin solution was removed and the gel pieces incubated with NH<sub>4</sub>HCO<sub>3</sub> (20 mM, 100 µL) at 37 °C overnight. The supernatant was collected in a fresh tube and the gel pieces were extracted with acetonitrile/TFA in water (0.1%, 1:1) twice for 60 min at rt and the supernatant was added to the previously collected. The extracted peptides were freeze-dried and subjected to mass spectrometric analysis with nano-LC-MS/MS.

### 2.3.7 *On-Beads Digestion*

The beads were further washed with NH<sub>4</sub>HCO<sub>3</sub> (25 mM, 2 x 1 mL) and incubated with DTT (10 mM) in NH<sub>4</sub>HCO<sub>3</sub> (25 mM, 1 mL) at 56 °C for 1 h. The samples were cooled to rt, the supernatant was removed and incubation followed with iodoacetamide (50 mM) in NH<sub>4</sub>HCO<sub>3</sub> (25 mM, 1 mL) at rt for 1 h in the dark. The supernatant was removed and the beads were washed with NH<sub>4</sub>HCO<sub>3</sub> (25 mM, 2 x 1 mL). Digestion followed with trypsin (10 µg/mL, *Promega*) and *RapiGest*™ SF (400 µg/mL, *Waters*) in NH<sub>4</sub>HCO<sub>3</sub> (25 mM, 600 µL) at 37 °C overnight. The reaction was stopped with TFA (1%) in MQ (1 mL) and the supernatant was transferred into a

fresh tube. The remaining beads were washed with TFA (0.1%) in MeCN/MQ 1:4 (2 x 600  $\mu$ L) and the supernatants were combined. The samples were lyophilized and desalted using U-C18 ZipTips (*Merck Millipore*) before injection into the mass spectrometer.

### 2.3.8 LC-MS/MS Analysis

Tryptic peptides were separated by reversed-phase nano-HPLC (*Eksigent*, 5  $\mu$ m, C18, 100 Å pore size, 75  $\mu$ m x 10 cm silica capillary (*Acclaim PepMap100*, *Thermo Scientific*)) using a washing step of 5% mobile phase B (0.1% formic acid in MeCN) in mobile phase A (0.1% formic acid) for 5 min, followed by a gradient from 5% mobile phase B to 40% mobile phase B in 65 min with a subsequent gradient to 80% mobile phase B in another 5 min at a flowrate of 300 nL/min. Each of the three independent biological replicates was measured as technical duplicate. Mass spectra were collected on an LTQ-Orbitrap mass spectrometer (*Thermo Fisher*) operated in data dependent mode with dynamic exclusion. Each full MS scan (30,000 resolving power) was followed by five MS/MS scans of the five most abundant molecular ions that were dynamically selected and fragmented by CID using a normalized collision energy of 35% in the LTQ ion trap.

Raw files from LC-MS/MS measurements were analyzed using *MaxQuant* (version 1.5.5.1) with default settings, match between runs and LFQ (minimum ratio count 2) enabled. For protein identification, the human reference proteome was downloaded from the *UniProt* database and an integrated database of common contaminants was used. Identified proteins were further processed using *Perseus* (version 1.5.8.5) and filtered for reverse hits and common contaminants at a false discovery rate of 0.01. LFQ intensities were further log<sub>2</sub> transformed. The proteins were filtered to be detected in at least four replicates among six replicate experiments and missing values were imputed from a normal distribution (width = 0.3, shift = 1.8), based on the assumption that these proteins were below the detection limit. Significantly enriched proteins were identified by a Student's t-test (FDR = 0.01, s0 = 2), comparing LFQ intensities of ABPP with probe C with the controls. The data was visualized in a volcano plot of log<sub>2</sub> fold change versus the negative logarithmized p-values. Further data analysis was performed with *Gene Ontology* (GO) annotation terms from *UniProt*.

In case of the *in-gel* digested samples MS/MS spectra were searched against the Swissprot human database using Mascot (*Matrix Science*) with "Trypsin/P" enzyme cleavage, static cysteine alkylation by iodoacetamide and variable methionine oxidation without further processing with *Perseus*.

### 2.3.9 Analysis of Protein Sequences with *Knime*®

Utilizing the *Knime*® software (expounded and set up by Alexander Fillbrunn, AG Berthold) the sequences of the identified proteins were incorporated from the *UniProt* homepage, analyzed in large-scale and compared to known sequence motifs of nucleotide binding proteins. Furthermore, GO terms were downloaded from the *UniProt* homepage for each respective protein, incorporated to the *Knime* platform and analyzed.

## 2.4 Assays Performed to Identify the Interactome of Ap<sub>3</sub>A

### 2.4.1 Analytical RP-HPLC Assay to Determine the Stability of the Synthesized Ap<sub>3</sub>A Analogues

Diadenosine triphosphates (100  $\mu$ M) were treated with or without Fhit (400 nM) or SVPD (1.0 mu/ $\mu$ L) in buffer (12.5  $\mu$ M DTT, 5 mM HEPES, 5 mM MgCl<sub>2</sub>, pH = 6.8) for 1 h at 30 °C in a total volume of 30  $\mu$ L. The reactions were quenched at 95 °C for 5 min and the protein content was removed with Vivaspin® 500 centrifugal concentrators (3000 MWCO, *Sartorius*) prior to analysis. HPLC was performed on a NUCLEODUR® C<sub>18</sub> Pyramid 4 x 250 mm column (*Macherey-Nagel*) with a gradient of 0 to 40% MeCN in TEAA buffer (50 mM triethylammonium acetate, pH 7.0) over 20 min.

### 2.4.2 FRET Assay to Determine the Competitive Potential of the Synthesized Ap<sub>3</sub>A Analogues with FRET-Ap<sub>3</sub>A

Different concentrations of the non-hydrolyzable Ap<sub>3</sub>A analogs (0 – 100  $\mu$ M) were incubated with Fhit (4 nM) in buffer T (12.5  $\mu$ M DTT, 5 mM HEPES, 5 mM MgCl<sub>2</sub>, pH = 7.0) for 30 min at rt. The reaction was started by addition of FRET-Ap<sub>3</sub>A (10  $\mu$ M) and the fluorescence intensity was measured every three minutes over 1 h at rt in an infinite F500 plate reader (*TECAN*).

### 2.4.3 General Procedure of Photoaffinity Labeling Between Proteins and Probe A

Fhit or BSA (1.0  $\mu$ M) and probe A (10  $\mu$ M) were incubated in sodium dihydrogen phosphate buffer (50 mM, 5 mM MgCl<sub>2</sub>, pH = 7.0) at rt for 30 min in a total volume of 20  $\mu$ L. The open

tubes were placed on ice and were irradiated with UV light at 366 nm for 60 min if not indicated otherwise. Controls were performed as indicated in the respective figures.

#### 2.4.4 General Procedure of CuAAC to N<sub>3</sub>-Cy5

The samples obtained from photoaffinity labeling were mixed with SDS (250  $\mu$ M), THPTA (10 mM), N<sub>3</sub>-Cy5 (12.5  $\mu$ M, 2.5 eq.) and started with the addition of Cu<sup>I</sup> (5 mM) in a total volume of 40  $\mu$ L for 1 h at rt. The reactions were quenched by the addition of SDS-PAGE loading dye (1x) and subjected to SDS-PAGE analysis, followed by optional WB analysis ( $\alpha$ -Fhit, 1:2000 in TNE-T, *abcam*).

#### 2.4.5 Optimization of Photoaffinity Labeling with Heparin or Polyacrylic Acid

The reactions were performed as in the general procedure, except probe A was added in 0.5  $\mu$ M concentration. Heparin (10  $\mu$ g/mL or 100  $\mu$ g/mL) and polyacrylic acid (0.5  $\mu$ M or 10  $\mu$ M) were added as indicated during the incubation step before photoaffinity labeling.

#### 2.4.6 Optimization of Photoaffinity Labeling with BSA, Different Azides and Strep-AP Read-out

The reactions were performed as in the general procedure, except probe A was added in 5.0  $\mu$ M concentration. CuAAC with different azides was performed similarly to the described procedure, except that 10  $\mu$ M of azides were applied. Read-out with Strep-AP was performed via transfer on a WB-membrane and development of nitro blue tetrazolium chloride (NBT, 330  $\mu$ L in 1 mL H<sub>2</sub>O), 5-bromo-4-chloro-3-indolyl phosphate (BCIP, 30  $\mu$ L in 1 mL DMF) and alkaline phosphatase (AP)-streptavidin conjugate (*Invitrogen*) in AP buffer (1 mL, 100 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, pH 9.5).

#### 2.4.7 Optimization of CuAAC Conditions after Photoaffinity Labeling

The photoreactions followed by CuAAC for the optimization of the utilized Cu salts, ligands and reducing agents were performed as described in the general procedures, except 5  $\mu$ M Fhit and 20  $\mu$ M probe A were applied. The Cu salts were used similarly to the reduction agents TCEP and sodium ascorbate in 5 mM concentration, N<sub>3</sub>-TAMRA-biotin was applied in 20  $\mu$ M.

For the optimization of CuAAC conditions with Cu<sup>I</sup> and THPTA, Fhit was applied in 1  $\mu$ M, probe A in 5  $\mu$ M and the reactions were performed as described in the general procedure. Cu<sup>I</sup>, THPTA and N<sub>3</sub>-biotin-azide were applied as indicated in the respective figure.

### 2.4.8 Optimization of UV Irradiation During Photoaffinity Labeling

The reactions were performed as described in the general procedures, except probe A was applied in 5  $\mu$ M. In the CuAAC, N<sub>3</sub>-TAMRA-biotin was applied in 5  $\mu$ M (2.0 eq.).

### 2.4.9 Photoaffinity Labeling, Followed by CuAAC and Binding to Streptavidin

The reactions were performed as described in the general procedures, except probe A was applied in 5  $\mu$ M. In the CuAAC, N<sub>3</sub>-TAMRA-biotin was applied in 10  $\mu$ M (4.0 eq.), streptavidin was added in 5  $\mu$ M.

### 2.4.10 ABPP in *E. coli* Cell Lysate Overexpressing Fhit with Probe A

*E. coli* BL21(DE3)RIL cells containing a vector for overexpression of Fhit, were grown overnight at 37 °C. The overnight culture was diluted 10-fold in fresh LB medium and was grown to an OD of 0.8 when IPTG (1 mM) was added. The cells were grown overnight at 37 °C and harvested by centrifugation at 4 °C (4000 rpm, 10 min). The pellet was frozen at –20 °C until use. *E. coli* cells were resuspended in lysis buffer (50 mM Tris-HCl, 20 mM MgCl<sub>2</sub>, 2 mM  $\beta$ -mercaptoethanol, 0.1% Triton-X-100, pH = 8.0) containing protease inhibitors Pefabloc® (1  $\mu$ g/mL), aprotinin (1  $\mu$ g/mL) and leupeptin (1  $\mu$ g/mL), disrupted by sonication (2 x 30 s, 5 x cycle, 20%) and the supernatant was cleared by centrifugation at 4 °C (14000 rpm, 30 min).

The photoreaction was carried out with different concentrations of probe A (0–50  $\mu$ M) following the general procedure with *E. coli* lysate (1.2 mg/mL). CuAAC was performed as described with N<sub>3</sub>-TAMRA-biotin (5.0  $\mu$ M, Jena Bioscience). Acetone (–20 °C, 150  $\mu$ L) was added and the tubes were left at 4 °C for 20 min before centrifugation at 4 °C (14.000 rpm, 10 min). The pellets were resuspended in MeOH (–20 °C, 150  $\mu$ L) by sonication and again subjected to centrifugation (14.000 rpm, 10 min). The supernatant was discarded and this step was repeated once. The pellet was resuspended in 1 x PBS containing 0.4% SDS (50  $\mu$ L) and incubated with streptavidin sepharose beads (15  $\mu$ L, GE Healthcare) that have been washed with 1 x PBS previously (3 x) for 1 h at rt. After incubation the supernatant was removed and the beads were washed with 1 x PBS, containing 0.4% SDS (3 x 100  $\mu$ L), urea (6 M, 2 x 100  $\mu$ L) and 1 x PBS (3 x 100  $\mu$ L) and centrifuged (rt, 2500 rpm, 3 min). The pellet was mixed with

1 x PBS buffer (50 mL) and SDS loading dye (6 x, 10  $\mu$ L) and heated to 95 °C for 5 min. The mixture was centrifuged (rt, 13400 rpm, 5 min), the supernatant transferred into a fresh tube and stored at –20 °C until analysis by SDS-PAGE.

#### 2.4.11 Optimization of ABPP in HEK 293T Cell Lysate with Probe A

In a total volume of 20  $\mu$ L probe A was applied in varying concentrations (0.0  $\mu$ M, 1.0  $\mu$ M, 5.0  $\mu$ M, 10  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 500  $\mu$ M). In a bigger batch, in a total volume of 200  $\mu$ L, probe A was added with 10  $\mu$ M or 100  $\mu$ M concentration. In a mixture with sodium dihydrogen phosphate buffer (50 mM, 5 mM MgCl<sub>2</sub>, pH = 7.0) and lysate (0.5 mg/mL) the samples were incubated for 30 min at rt before UV irradiation at 4 °C for 60 min. In a total volume of 40  $\mu$ L or 400  $\mu$ L, respectively, the mixture was reacted with N<sub>3</sub>-TAMRA-biotin (2.0 eq.) in SDS (250  $\mu$ M), THPTA (5 mM) and Cu<sup>I</sup> (5 mM) for 60 min at rt. The reaction was mixed with ice cold acetone (40  $\mu$ L or 400  $\mu$ L), incubated at 4 °C for 20 min, centrifuged (4 °C, 14000 rpm, 10 min) and the pellet was stored at –20 °C. After resuspension by sonication with MeOH (40  $\mu$ L or 400  $\mu$ L), the sample was centrifuged (4 °C, 14000 rpm, 10 min) and this procedure was repeated. The pellet was then resuspended in 1 x PBS, containing 0.4% SDS (40  $\mu$ L or 400  $\mu$ L) and incubated with sepharose streptavidin beads (5  $\mu$ L or 20  $\mu$ L) for 60 min at rt. The sepharose streptavidin beads were washed with 1 x PBS (3 x 200  $\mu$ L) and centrifuged (rt, 2500 rpm, 3 min) before use. After incubation the supernatant was removed and the beads were washed with 1 x PBS, containing 0.4% SDS (3 x 50  $\mu$ L), urea (6 M, 2 x 50  $\mu$ L) and 1 x PBS (3 x 50  $\mu$ L) and centrifuged (rt, 2500 rpm, 3 min). The pellet was mixed with 1 x PBS buffer (35  $\mu$ L) and SDS loading dye (6x, 7  $\mu$ L) and heated to 95 °C for 5 min. The mixture was centrifuged (rt, 13400 rpm, 5 min), the supernatant transferred into a fresh tube and stored at –20 °C until analysis with SDS-PAGE.

Control experiments were performed as follows. In a total volume of 100  $\mu$ L lysate (1.0 mg/mL) was treated with UV light as described before, followed by incubation with sepharose streptavidin beads (30  $\mu$ L) and work-up as presented earlier. One sample was not treated with beads. An additional step of CuAAC with different concentrations of N<sub>3</sub>-TAMRA-biotin (20  $\mu$ M or 500  $\mu$ M) was implemented with and without acetone precipitation and beads incubation. These samples did not contain any Ap<sub>3</sub>A.

Further controls included probe A (50  $\mu$ M) and lysate (1.0 mg/mL) and N<sub>3</sub>-TAMRA-biotin (100  $\mu$ M) in the CuAAC. They were performed without beads incubation or acetone precipitation but with and without Cu<sup>I</sup> and THPTA during CuAAC. Furthermore, a heat control (95 °C, 5 min, 2% SDS before addition of probe A) and a no UV light control was performed.

#### **2.4.12 ABPP in HEK 293T Cell Lysate with Probe A, Competitor B and N<sub>3</sub>-TAMRA-Biotin (analytical)**

In a total volume of 100  $\mu$ L probe A (250  $\mu$ M) was mixed with sodium dihydrogen phosphate buffer (50 mM, 5 mM MgCl<sub>2</sub>, pH = 7.0) and lysate (1 mg/mL) and was incubated for 30 min at rt. At 4 °C the open tube was positioned under a UV lamp (366 nm) for 60 min. In a total volume of 150  $\mu$ L, the mixture was reacted with N<sub>3</sub>-TAMRA-biotin (250  $\mu$ M), SDS (250  $\mu$ M), THPTA (5 mM) and Cu<sup>I</sup> (5 mM) for 60 min at rt. The reaction was mixed with ice cold acetone (150  $\mu$ L), incubated at 4 °C for 20 min, centrifuged (4 °C, 14000 rpm, 10 min) and the pellet was stored at -20 °C. After resuspension by sonication with MeOH (100  $\mu$ L), the sample was centrifuged (4 °C, 14000 rpm, 10 min) and this procedure was repeated. The pellet was resuspended in 1 x PBS, containing 0.4% SDS (50  $\mu$ L) and incubated with sepharose streptavidin beads (85  $\mu$ L) for 60 min at rt. The sepharose streptavidin beads were washed with 1 x PBS (3 x 200  $\mu$ L) and centrifuged (rt, 2500 rpm, 3 min) before use. After incubation the supernatant was removed and the beads were washed with 1 x PBS, containing 0.4% SDS (3 x 100  $\mu$ L), urea (6 M, 2 x 100  $\mu$ L) and 1 x PBS (3 x 100  $\mu$ L) and centrifuged (rt, 2500 rpm, 3 min). The pellet was mixed with 1 x PBS buffer (50 mL) and SDS loading dye (6 x, 10  $\mu$ L) and heated to 95 °C for 5 min. The mixture was centrifuged (rt, 13400 rpm, 5 min), the supernatant transferred into a fresh tube and stored at -20 °C until analysis with SDS-PAGE.

The same procedure was conducted when a 4-fold excess of probe B (1 mM) was incubated with cell lysate for 30 min at rt, before probe A (250  $\mu$ M) was added and the UV irradiation was started.

#### **2.4.13 ABPP in HEK 293T Cell Lysate with Probe A, Competitor B and N<sub>3</sub>-TAMRA-Biotin or N<sub>3</sub>-Biotin (preparative)**

The preparative experiment was performed similarly to the analytical experiment, followed by *in-gel* digestion of the respective proteins bands or complete lanes. Additionally, experiments with CuAAC to N<sub>3</sub>-biotin were performed, similarly to CuAAC with N<sub>3</sub>-TAMRA-biotin.

#### **2.4.14 General Procedure of Photoaffinity Labeling of Proteins by Probe C**

Probe C (10  $\mu$ M) was incubated with Fhit or BSA (5.0  $\mu$ M) in phosphate buffer (50 mM, 5 mM MgCl<sub>2</sub>, pH 7.0) for 30 min at rt and irradiated for 45 min at 366 nm with open lid on ice, if not indicated otherwise. The samples were diluted with SDS-PAGE loading dye (1x) and subjected to SDS-PAGE and/or WB analysis (ExtrAvidin-HRP, *sigma-aldrich*, 1:10.000 in TNE-T) or directly analyzed by HR-ESI-MS.

The competition experiments were performed with probe B in different concentrations (0.0  $\mu\text{M}$ , 10  $\mu\text{M}$ , 50  $\mu\text{M}$ , 100  $\mu\text{M}$ , 500  $\mu\text{M}$ , 1.0 mM) whereby, probe C was first incubated with Fhit for 30 min at rt, before probe C was added, followed by UV irradiation.

#### 2.4.15 ABPP in HEK 293T Cell Lysate with Probe C (analytical)

In a total volume of 100  $\mu\text{L}$  probe C (250  $\mu\text{M}$ ) was mixed with sodium dihydrogen phosphate buffer (50 mM, 5 mM  $\text{MgCl}_2$ , pH 7.0) and freshly prepared lysate (1 mg/mL) and was incubated for 30 min at rt. At 4  $^{\circ}\text{C}$  the open tube was positioned under a UV lamp (366 nm) for 45 min. The samples were then incubated with streptavidin-coated sepharose beads (85  $\mu\text{L}$ ), previously washed with 1 x PBS (3 x 600  $\mu\text{L}$ ), for 1 h at rt. After incubation, the supernatant was removed and the beads were washed with 1 x PBS containing 0.4% SDS (3 x 600  $\mu\text{L}$ ), urea (6 M, 2 x 600  $\mu\text{L}$ ) and 1 x PBS (3 x 600  $\mu\text{L}$ ) and centrifuged (rt, 2500 rpm, 3 min). The samples were diluted with 2 x SDS-PAGE loading dye (6x, 14  $\mu\text{L}$ ) and MQ (35  $\mu\text{L}$ ) and heated for 5 min at 95  $^{\circ}\text{C}$ . After centrifugation (rt, 13,400 rpm, 5 min) the supernatant was collected in a fresh tube and stored at -20  $^{\circ}\text{C}$  until analysis with SDS-PAGE and WB (ExtrAvidin-HRP, *sigma-aldrich*, 1:10,000 in TNE-T).

A negative (beads) control without probe C was performed similarly to ABPP with probe C. Additionally, a heat control was conducted in which the lysate was pre-heated to 95  $^{\circ}\text{C}$  with 2% SDS before cooling down and addition of probe C. The competition experiment was performed by first incubating competitor probe B (2.5 mM) with lysate for 30 min at rt before addition of probe C (250  $\mu\text{M}$ ).

#### 2.4.16 ABPP in HEK 293T Cell Lysate with Probe C (preparative)

In a total volume of 1 mL probe C (250  $\mu\text{M}$ ) was mixed with sodium dihydrogen phosphate buffer (50 mM, 5 mM  $\text{MgCl}_2$ , pH 7.0) and freshly prepared lysate (1 mg/mL) and was incubated for 30 min at rt. At 4  $^{\circ}\text{C}$  the open tube was positioned under a UV lamp (366 nm) for 45 min. The samples were then incubated with streptavidin-coated sepharose beads (833  $\mu\text{L}$ , *GE Healthcare*), that were previously washed with 1 x PBS (3 x 1 mL), for 1 h at rt. After incubation the supernatant was removed and the beads were washed with 1 x PBS containing 0.4% SDS (3 x 1 mL), urea (6 M, 2 x 1 mL) and 1 x PBS (3 x 1 mL) and centrifuged (rt, 2500 rpm, 3 min). The samples were directly subjected to *on-beads* digestion.

Control experiments were performed similarly to the analytical experiment.

## 2.5 Assays Performed with Fhit Inhibitors

### 2.5.1 High-Throughput Screening

In a high throughput fashion, 15,136 compounds were screened for their activity against the Fhit protein in 384-well plates. For this purpose, the respective compounds (10  $\mu$ M) in DMSO were added to Fhit (6 nM, 50  $\mu$ L) in buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, 0.2 mg/mL, pH 8.0) to a final concentration of 1% DMSO. The mixtures were incubated at 25  $^{\circ}$ C for 30 min and the doubly labeled FRET-Ap<sub>3</sub>A probe (500 nM) was added in buffer (20 mM Tris-HCl, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, pH 8.0). As a negative control one lane in each plate was filled without enzyme and compound, only 1% DMSO and buffer. The positive control contained enzyme, 1% DMSO and no compound. The fluorescence intensity was measured immediately after addition of FRET-Ap<sub>3</sub>A at 535 nm excitation and 590 nm emission and again after incubation at 25  $^{\circ}$ C for 30 min. To ensure stability, FRET-Ap<sub>3</sub>A was stored in the dark at 4  $^{\circ}$ C until use. The residual enzymatic activity of Fhit was calculated for each compound relative to the respective negative and positive control on the same plate as the difference of the two measuring points. The data was obtained using a *TECAN* Infinite F500 and analyzed with the *KNIME*<sup>®</sup> Analytics Platform. The average z-score<sup>259</sup> was calculated as  $0.860 \pm 0.032$ , the SSMD value<sup>260</sup> as  $-27.244 \pm 6.572$ .

### 2.5.2 Dose-Response FRET Assay

To obtain the inhibitory potency of each individual derivative, a FRET assay was conducted with different concentrations of inhibitor. Therefore, the optimal Fhit concentration at which FRET-Ap<sub>3</sub>A was cleaved linearly over time needed to be adjusted. Concentrations of Fhit between 0 and 8 nM were tested and 1 nM was found to be suitable. In the assay Fhit (1 nM) in buffer (50 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) was mixed with assay buffer (5 mM HEPES, 2 mM DTT, 5 mM MgCl<sub>2</sub>, 0.1 mg/mL BSA, pH 6.8) and different concentrations of the respective compounds (5.08 nM, 15.2 nM, 45.7 nM, 137 nM, 412 nM, 1.23  $\mu$ M, 3.70  $\mu$ M, 11.1  $\mu$ M, 33.3  $\mu$ M and 100  $\mu$ M) in a final DMSO concentration of 1% in a total volume of 60  $\mu$ L. The mixtures were incubated for 30 min at 25  $^{\circ}$ C, doubly fluorescently labeled Ap<sub>3</sub>A (500 nM, 20  $\mu$ L) was added and the fluorescence intensity was measured every 3 min over 60 min. A positive (without inhibitor but 1% DMSO) and a negative control (without Fhit but respective buffer and the highest concentration of inhibitor) were conducted for each experiment. The inhibition in percent was calculated relative to the slope of fluorescence intensity of the positive control and relative *IC*<sub>50</sub> values<sup>261</sup> are presented. The data was obtained using a *TECAN* Infinite F500. All experiments were carried out in triplicates and the obtained data represent mean  $\pm$  standard deviation. All compounds were soluble in DMSO up to 20 mM concentration.

### 2.5.3 Cell Culture and MTT Assay<sup>256</sup>

HEK 293T and H1299 cells were maintained in DMEM supplement with 10% fetal bovine serum and penicillin/streptomycin at 37 °C with 5% CO<sub>2</sub> in the headspace. Cells were grown to 90% confluence and resuspended in 10 mL fresh DMEM. 28 µL of cells were mixed with 170 µL fresh DMEM and transferred to a 96-well plate. In a negative control only 198 µL of DMEM were used. The cells were grown at 37 °C overnight. Different concentrations of each inhibitor were added in 2 µL (5.08 nM, 15.2 nM, 45.7 nM, 137 nM, 412 nM, 1.23 µM, 3.70 µM, 11.1 µM, 33.3 µM and 100 µM) except for the negative control without cells and the positive controls with cells but without inhibitor in each of which 2 µL of DMSO was added. The cells were then incubated at 37 °C for 24 h. The medium was removed from the plates and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL, 100 µL) was added in DMEM and incubated at 37 °C for 1 h. The medium was removed again and DMSO (100 µL) was added and incubated at room temperature for 1 h. The absorbance was measured with a VICTOR<sup>3</sup> Multilabel Reader 1420 by *PerkinElmer* at 570 nm. Cell viability was calculated relative to the intensity of the positive and negative controls. All experiments were conducted in triplicates and data represent mean ± standard deviation.

### 2.5.4 HPLC Assay with Natural Ap<sub>3</sub>A

Fhit (400 nM) in protein buffer (50 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) and **19b** in DMSO were incubated in assay buffer (20 mM HEPES, 1 mM MgCl<sub>2</sub>, pH 7.2) in a total volume of 30 µL. Controls without Fhit and **19b** but protein buffer and DMSO and without **19b** but DMSO were conducted. The samples were incubated at 37 °C for 10 min and the reactions were started by the addition of natural Ap<sub>3</sub>A (200 µM, *Jena Bioscience*). After 1 h at 37 °C Fhit was inactivated by heating to 95 °C for 5 min. Protein was removed by VIVASPIN 500, 3.000 MWCO PES (*Sartorius*) prior to analysis. HPLC was performed on a NUCLEODUR® C<sub>18</sub> Pyramid 4 x 250 mm column (*Macherey-Nagel*) with a gradient of 0 to 40% MeCN in TEAA buffer (50 mM triethylammonium acetate, pH 7.0) over 20 min.

### 2.5.5 FRET Assay to Determine Reversibility of Inhibitor **19b**

Fhit (1 nM) in buffer (50 mM Tris-HCl, 1.0 mM EDTA, pH 8.0) was preincubated with **19b** (50 nM) in a final DMSO concentration of 1% in assay buffer (5 mM HEPES, 2 mM DTT, 5 mM MgCl<sub>2</sub>, 0.1 mg/mL BSA, pH 6.8) at 25 °C for 30 min. Increasing concentrations of FRET-Ap<sub>3</sub>A (0.1 µM, 0.5 µM, 2.5 µM and 12.5 µM) were added and the fluorescence intensity was measured every 3 min over 60 min. A positive control (without inhibitor but 1% DMSO) and a negative

control (without Hint but respective buffer and compound **19b**) were conducted for each substrate concentration. The data was obtained using a *TECAN* Infinite F500. All experiments were carried out in triplicates and the obtained data represent mean  $\pm$  standard deviation.

### 2.5.6 HPLC Assay to Determine Hint1 Inhibition by **19b**

Hint1 (400 nM, *abcam*) in protein buffer (20 mM Tris-HCl, 10% glycerol, pH 8.0) and **19b** in DMSO were incubated in assay buffer (20 mM HEPES, 1 mM MgCl<sub>2</sub>, pH 7.2) in a total volume of 30  $\mu$ L. Controls without Hint1 and **19b** but protein buffer and DMSO and without **19b** but DMSO were conducted. The samples were incubated at 37 °C for 10 min and the reactions were started by the addition of AMP-morpholidate (200  $\mu$ M, provided by Lena Krüger). After 1 h at 37 °C Hint1 was inactivated by heating to 95 °C for 5 min. Protein was removed by Vivaspin® 500 centrifugal concentrators (3000 MWCO, *Sartorius*) prior to analysis. HPLC was performed on a NUCLEODUR® C<sub>18</sub> Pyramid 4 x 250 mm column (*Macherey-Nagel*) with a gradient of 0 to 40% MeCN in TEAA buffer (50 mM triethylammonium acetate, pH 7.0) over 20 min.

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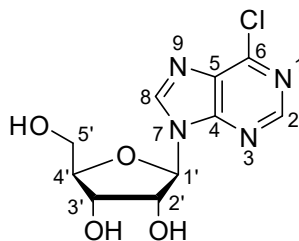
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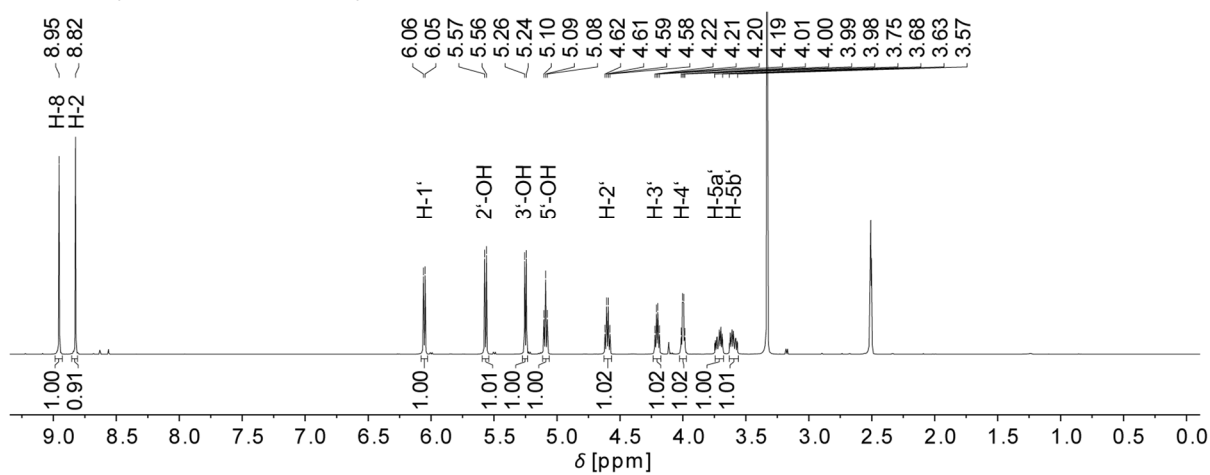


## VIII Appendix

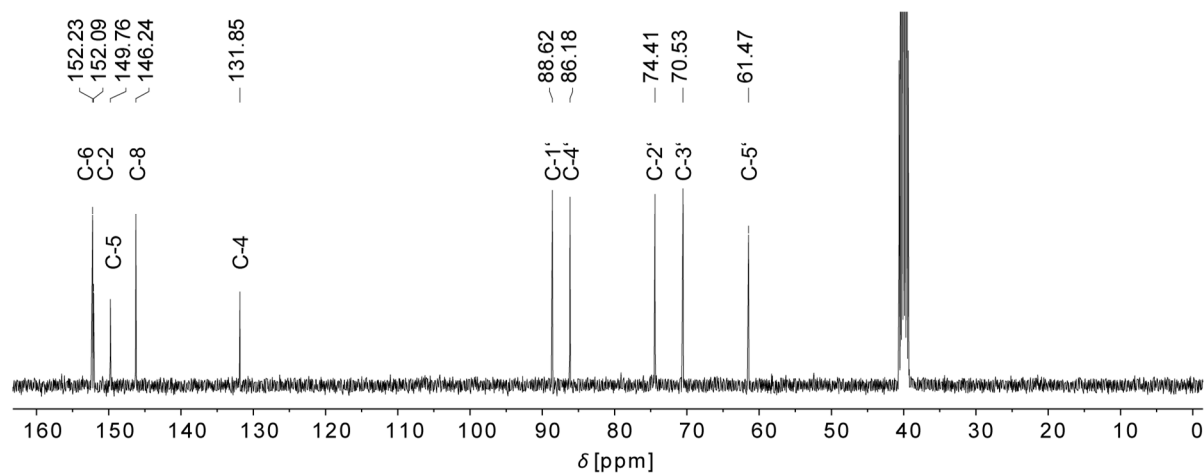
### 1 NMR Spectra of All Compounds

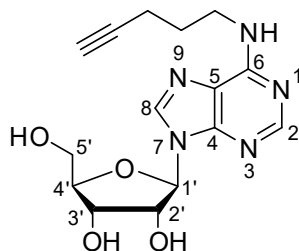
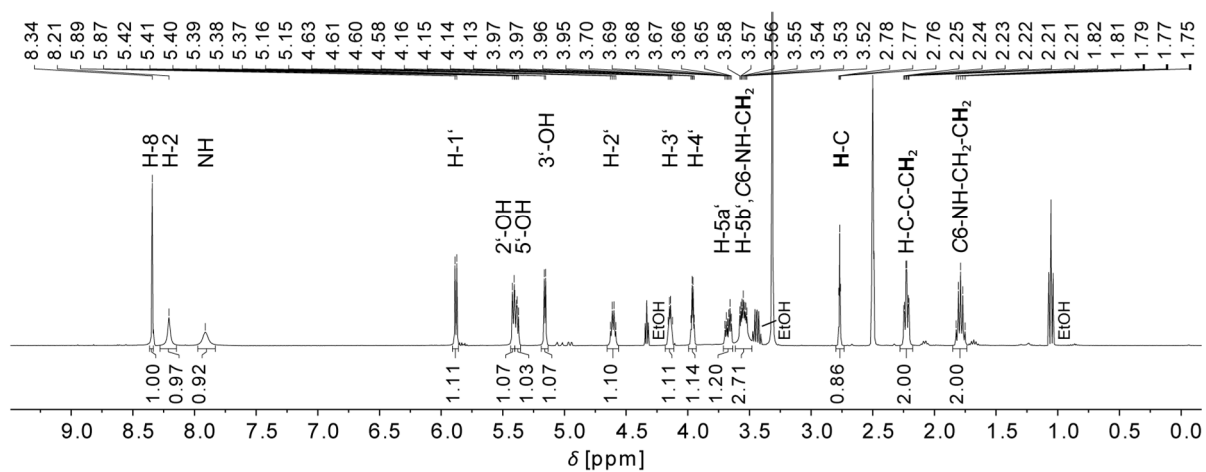
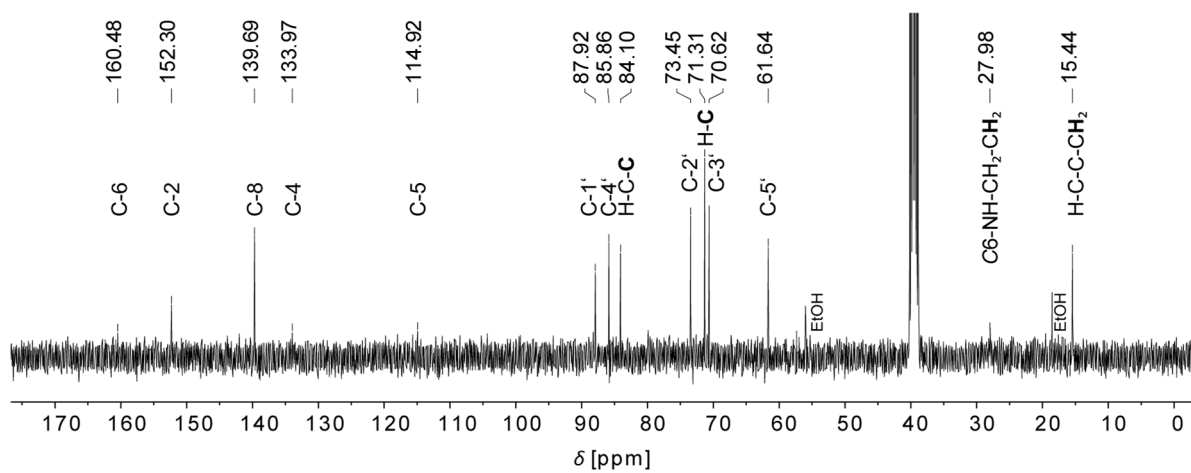


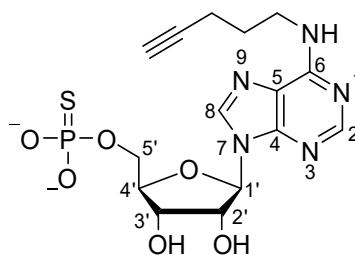
$^1\text{H}$  NMR (DMSO- $d_6$ , 400 MHz)



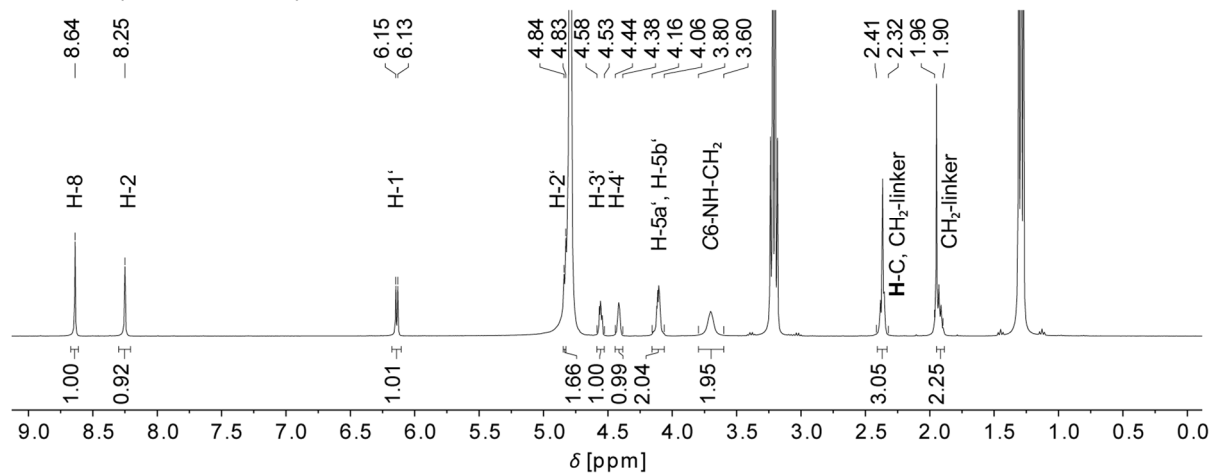
$^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz)



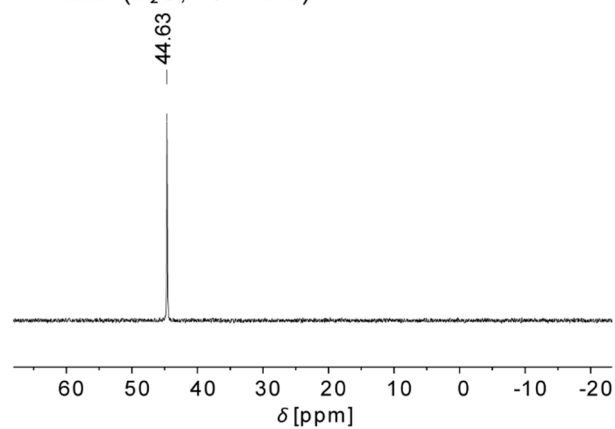
**<sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz)****<sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz)**

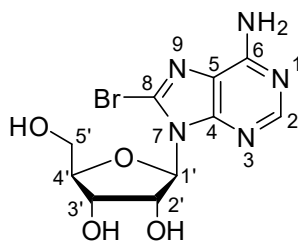


**$^1\text{H}$  NMR** ( $\text{D}_2\text{O}$ , 400 MHz)

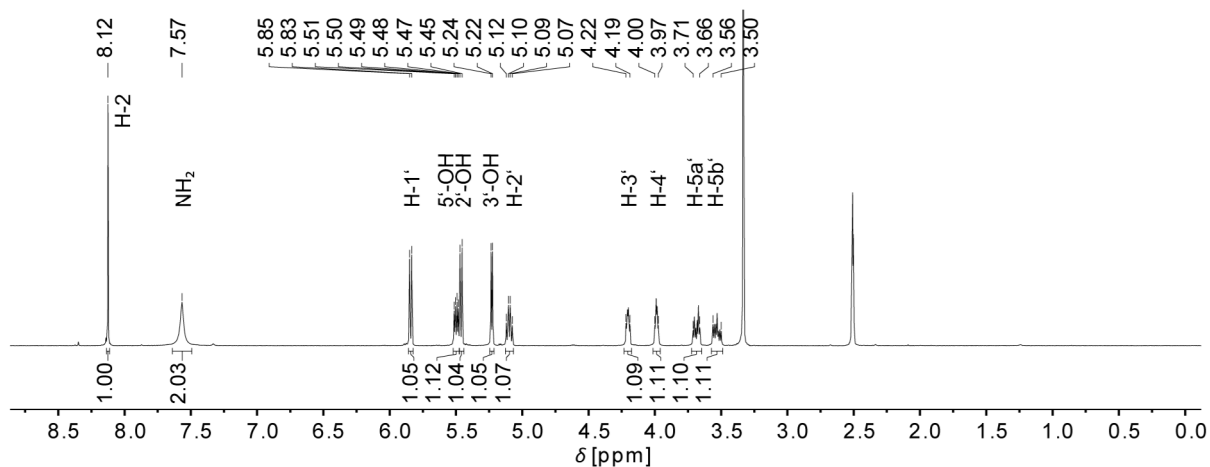


**$^{31}\text{P}$  NMR** ( $\text{D}_2\text{O}$ , 161 MHz)

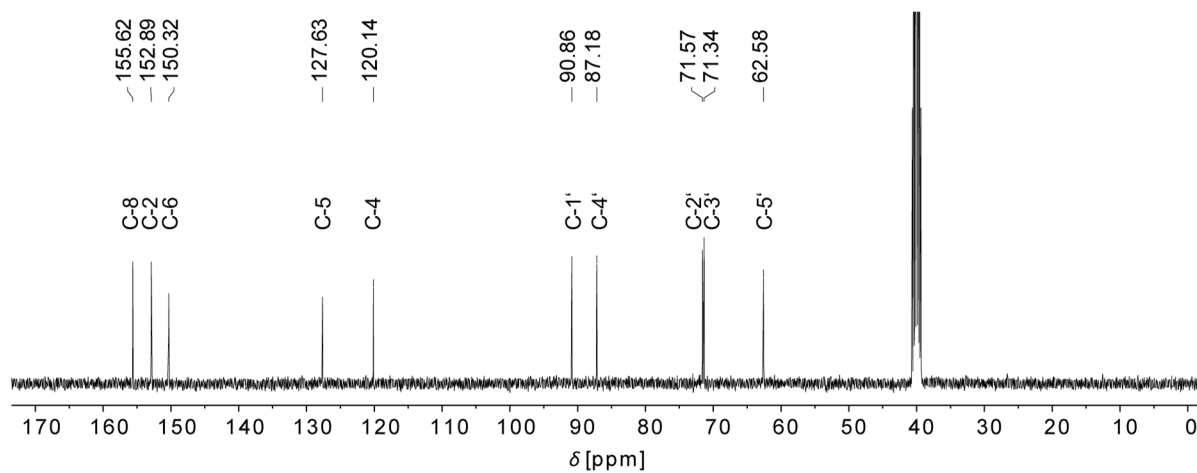


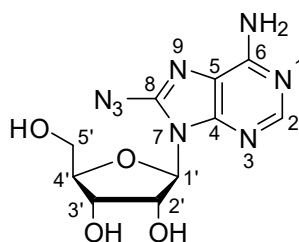


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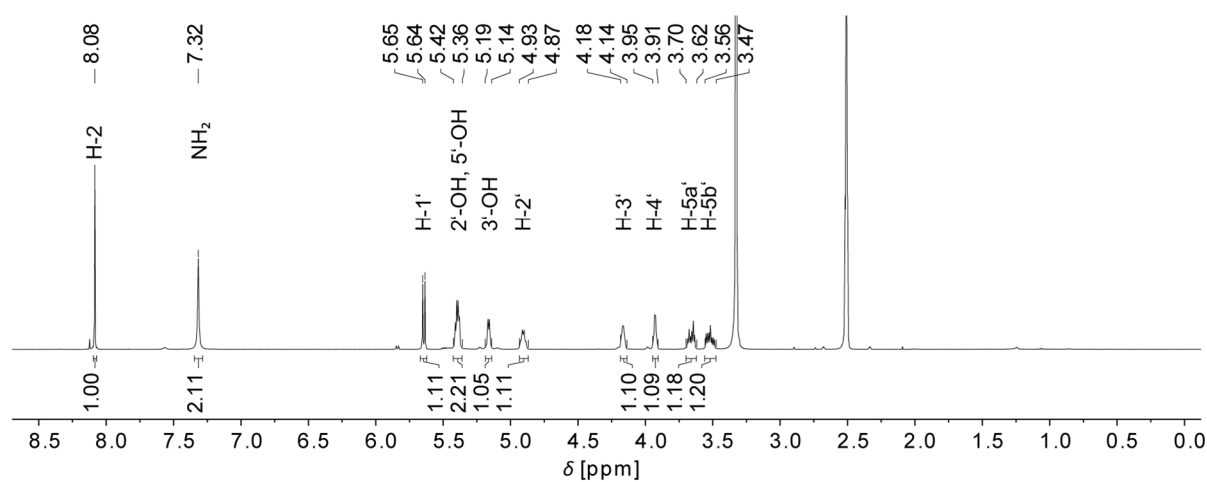


$^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz)

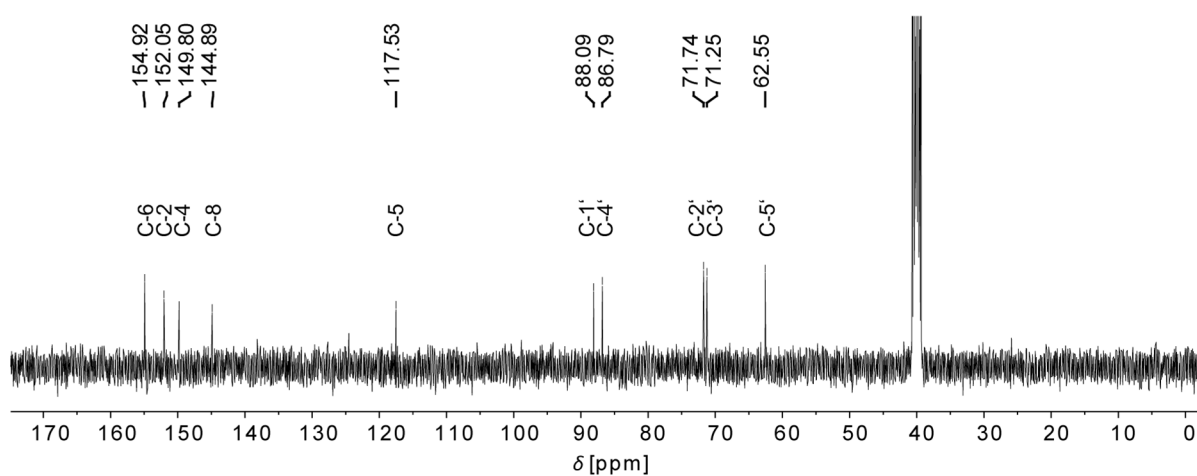


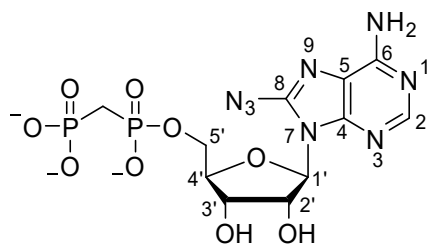
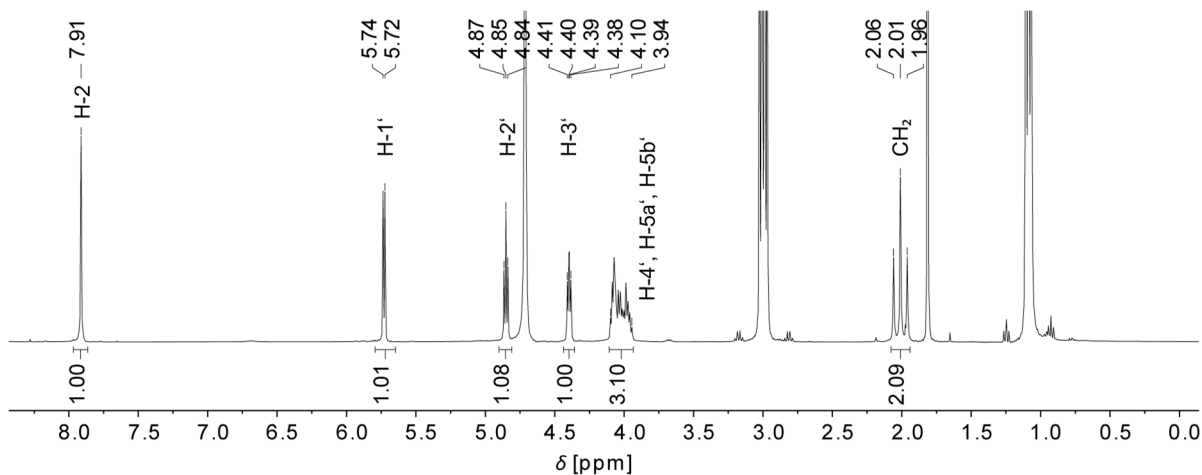
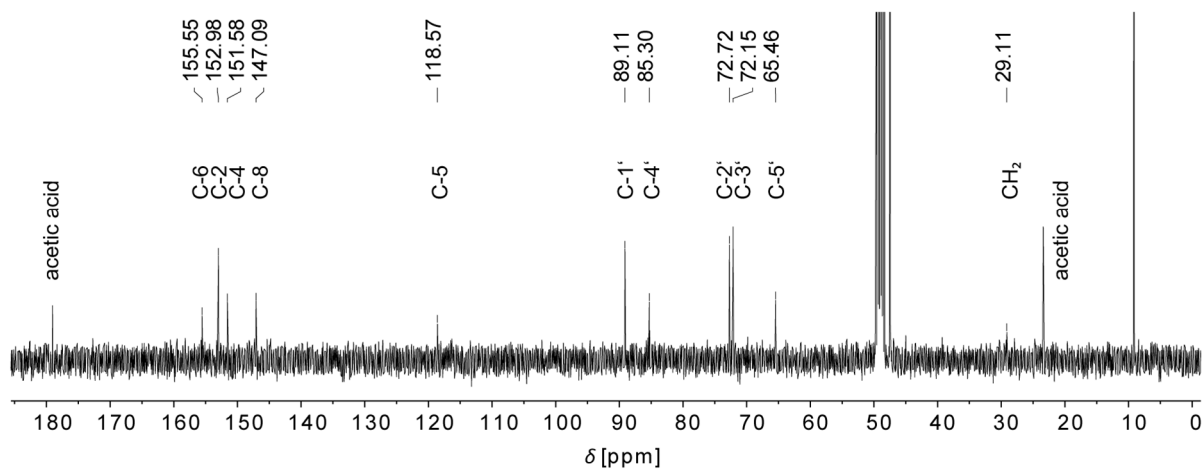
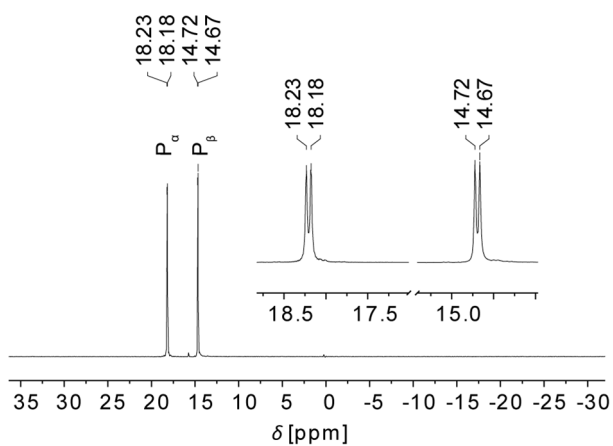


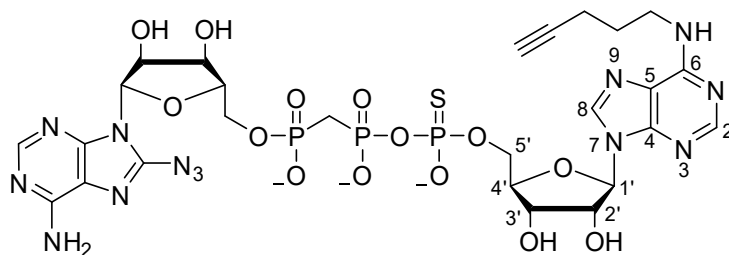
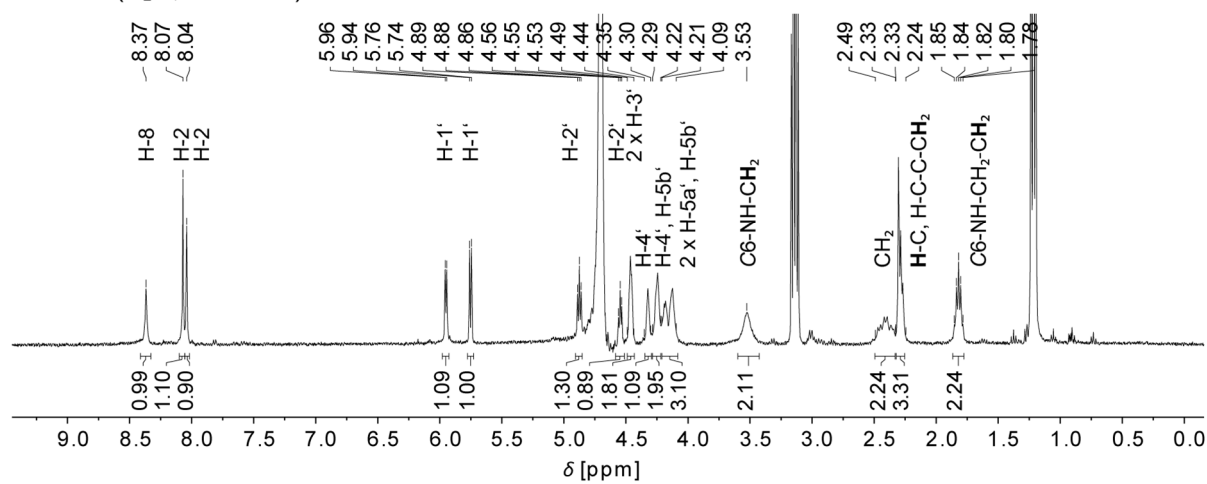
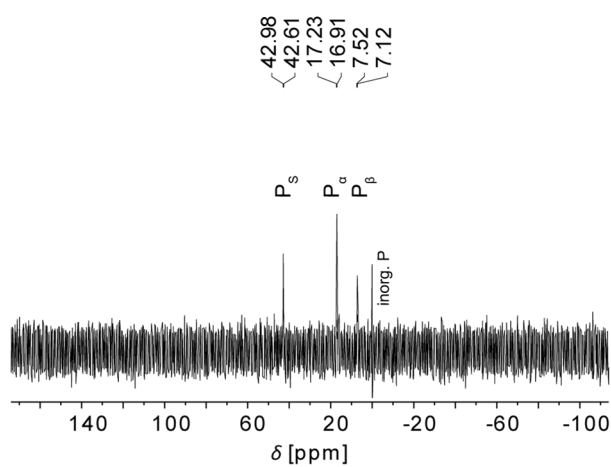
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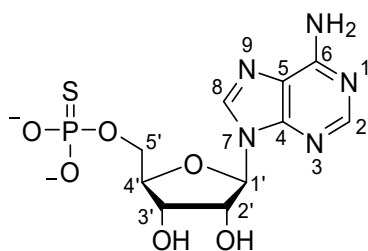


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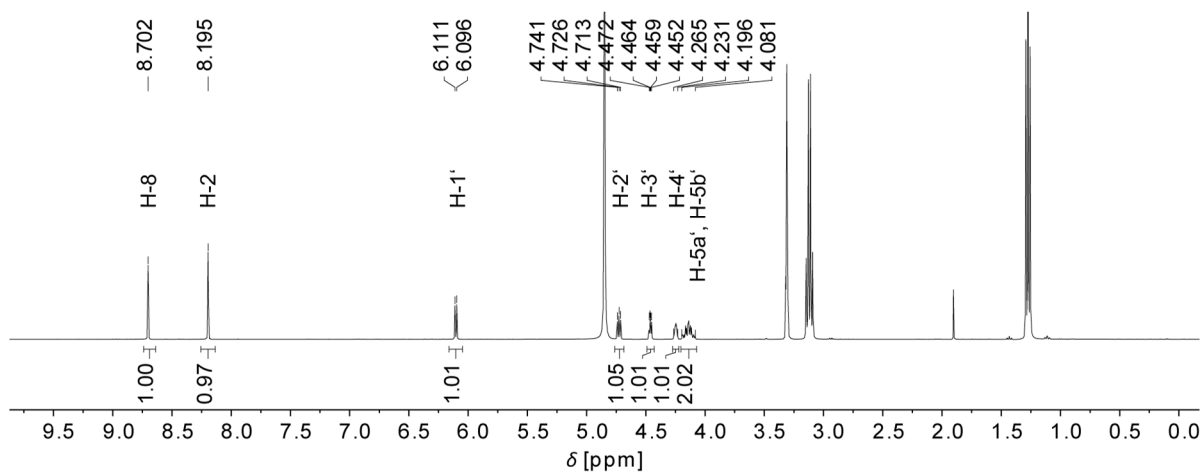


**<sup>1</sup>H NMR** (D<sub>2</sub>O, 400 MHz)**<sup>13</sup>C NMR** (MeOD, 100 MHz)**<sup>31</sup>P NMR** (D<sub>2</sub>O, 161 MHz)

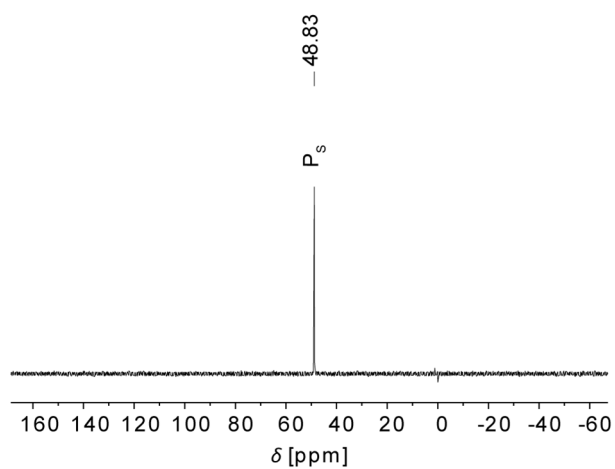
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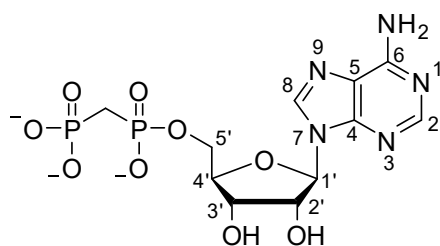


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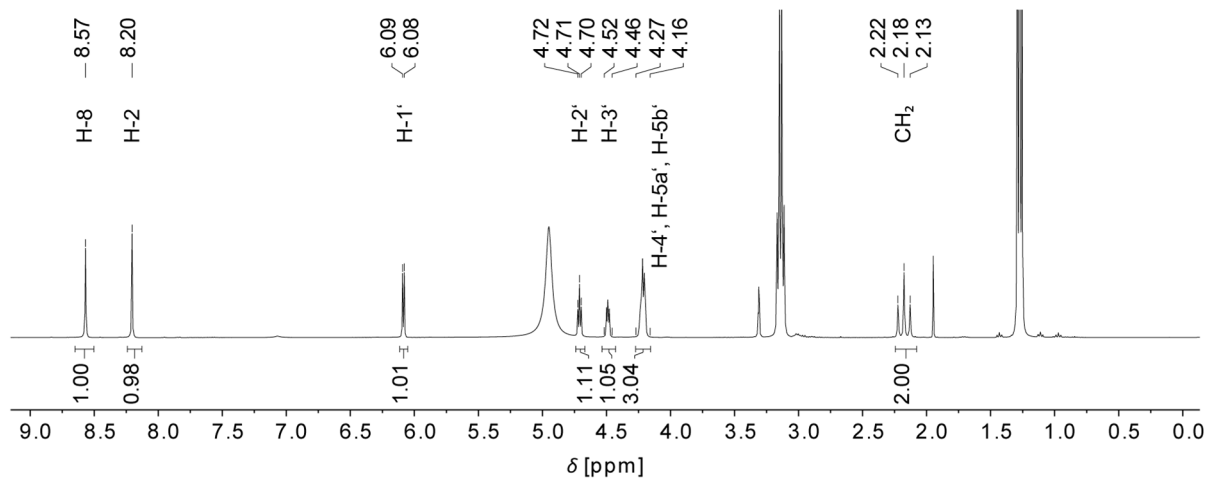


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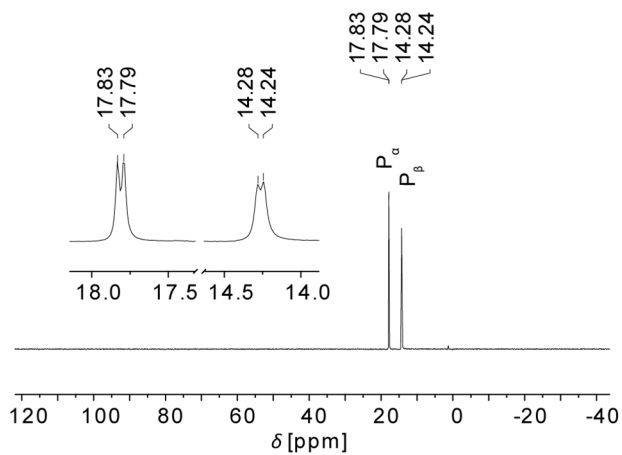


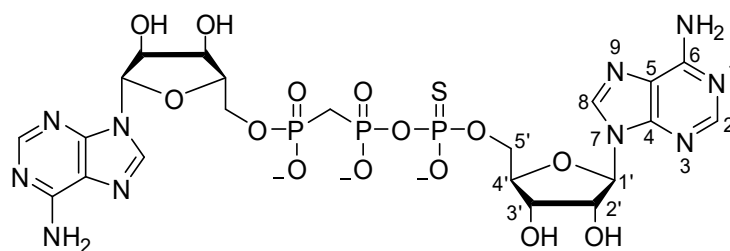
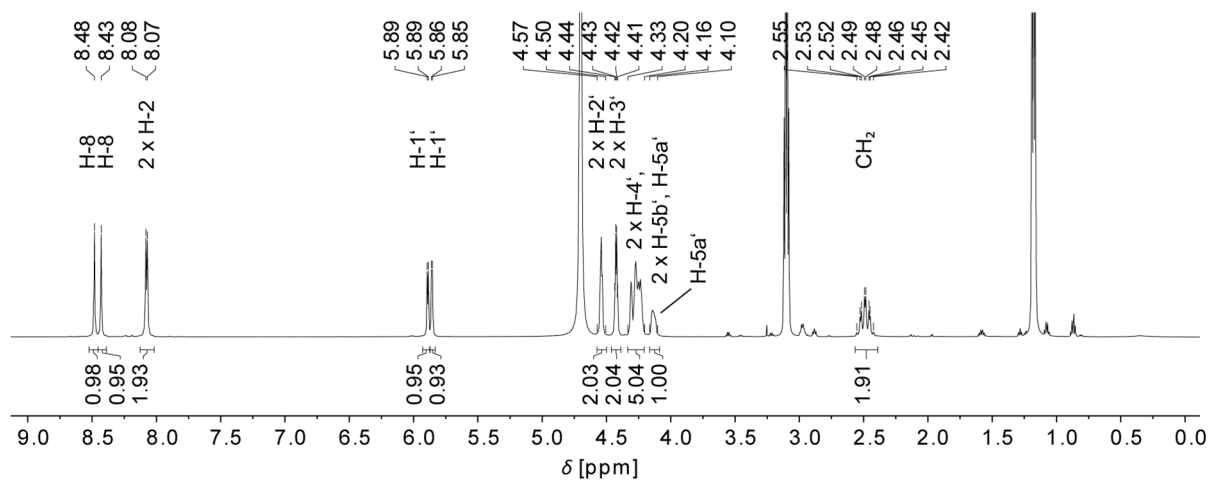
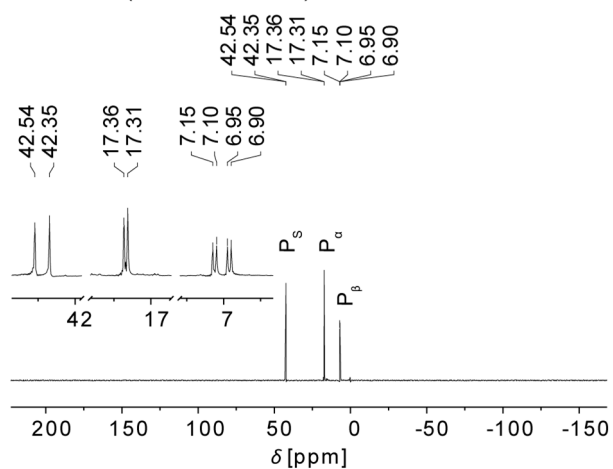


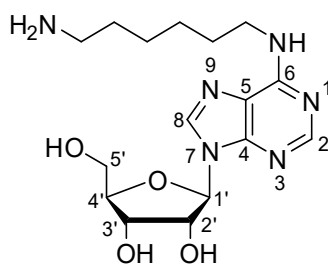
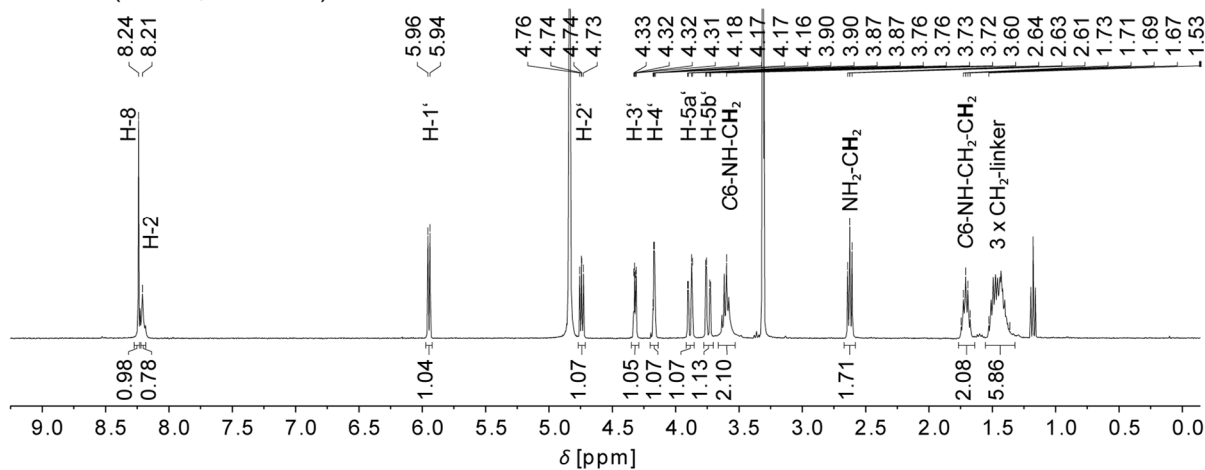
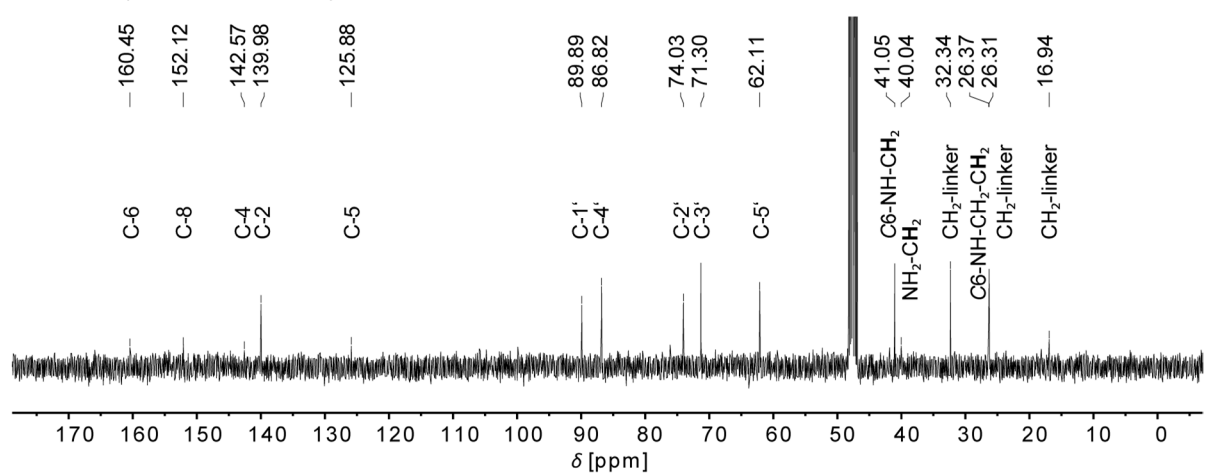
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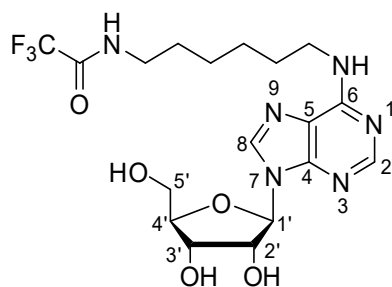
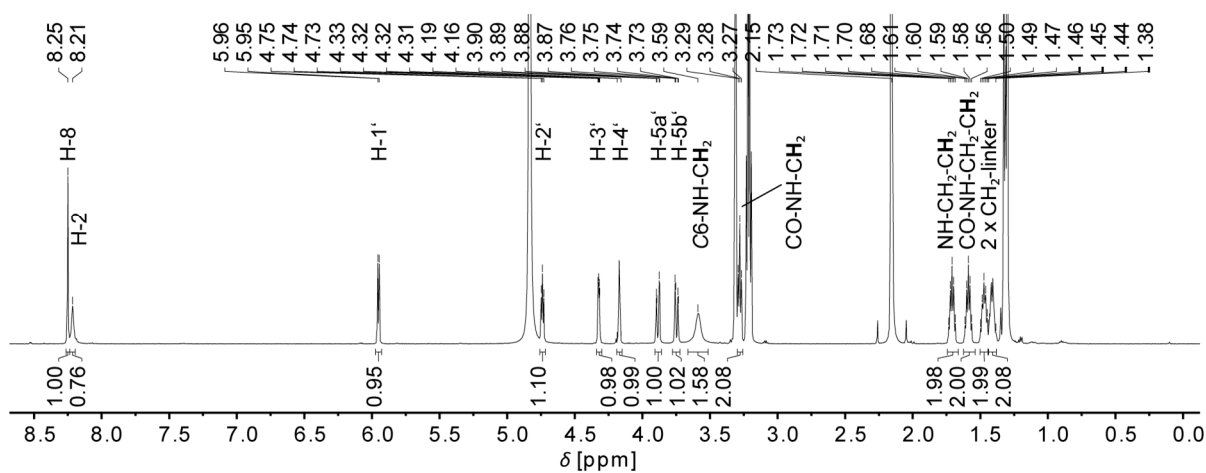
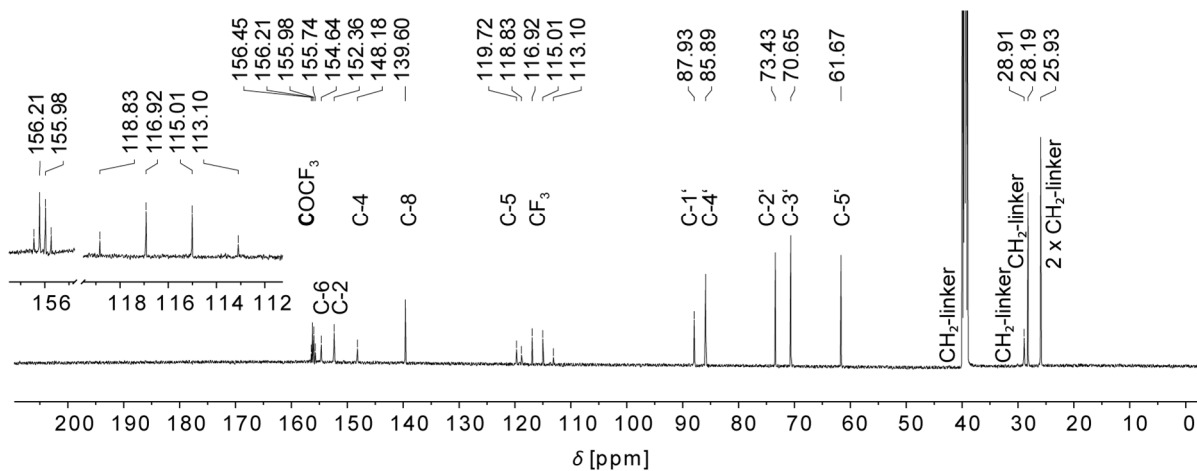
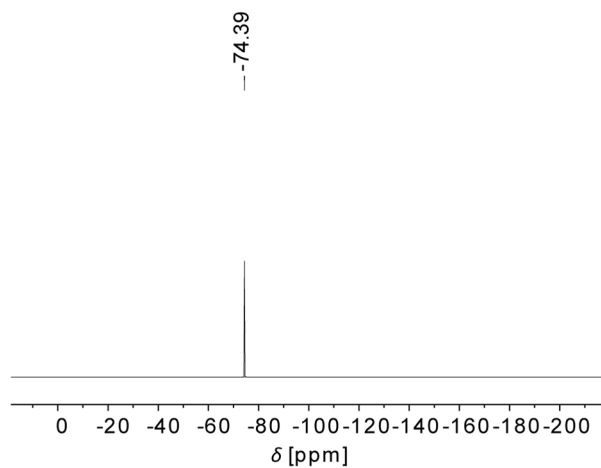


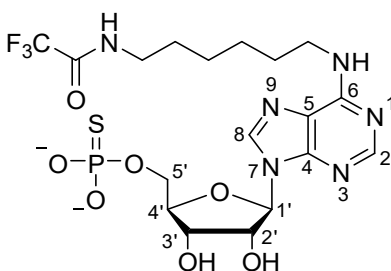
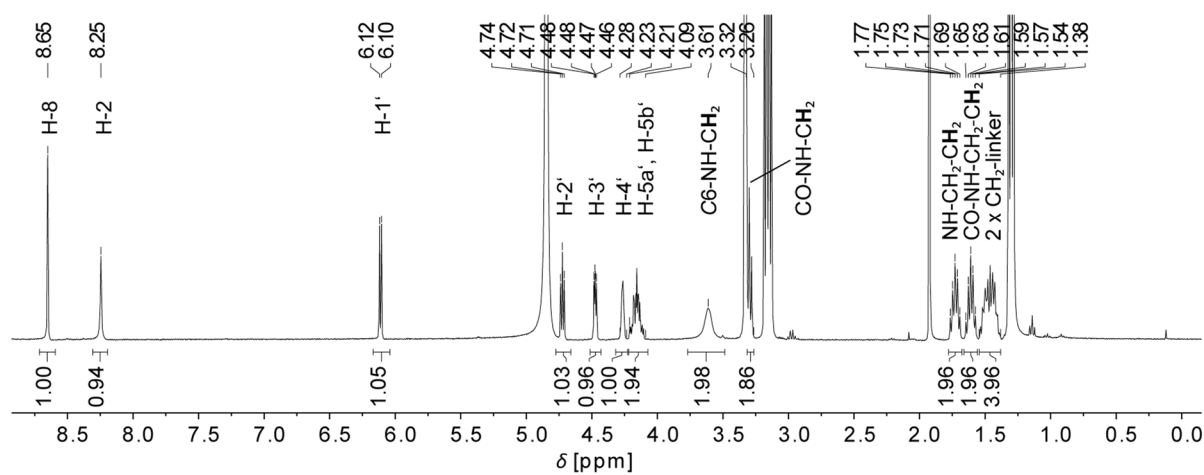
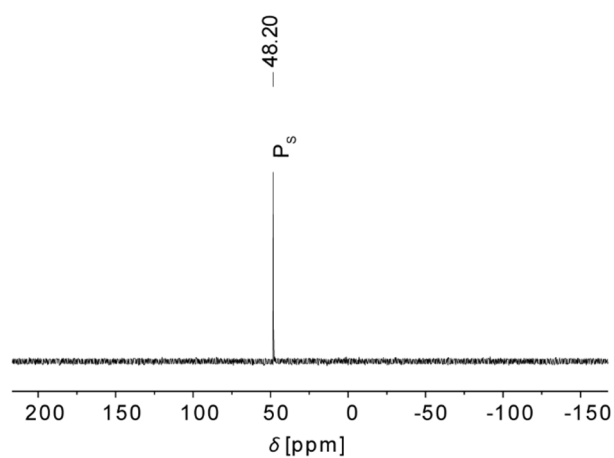
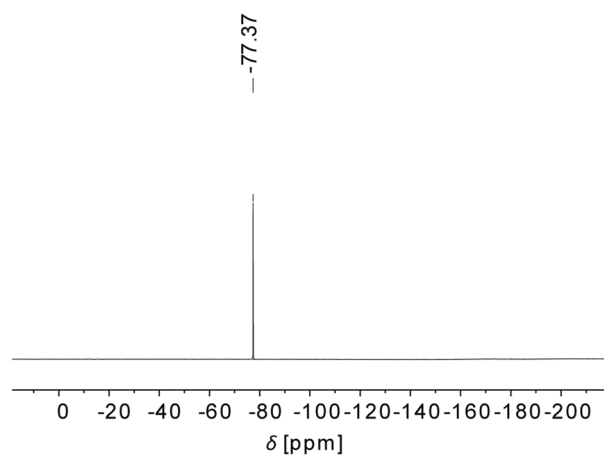
**<sup>31</sup>P NMR** (MeOD, 161 MHz)

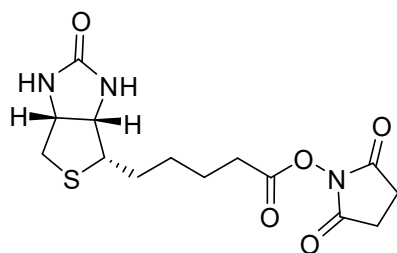


**<sup>1</sup>H NMR** (D<sub>2</sub>O, 400 MHz)**<sup>31</sup>P NMR** (D<sub>2</sub>O, 161 MHz)

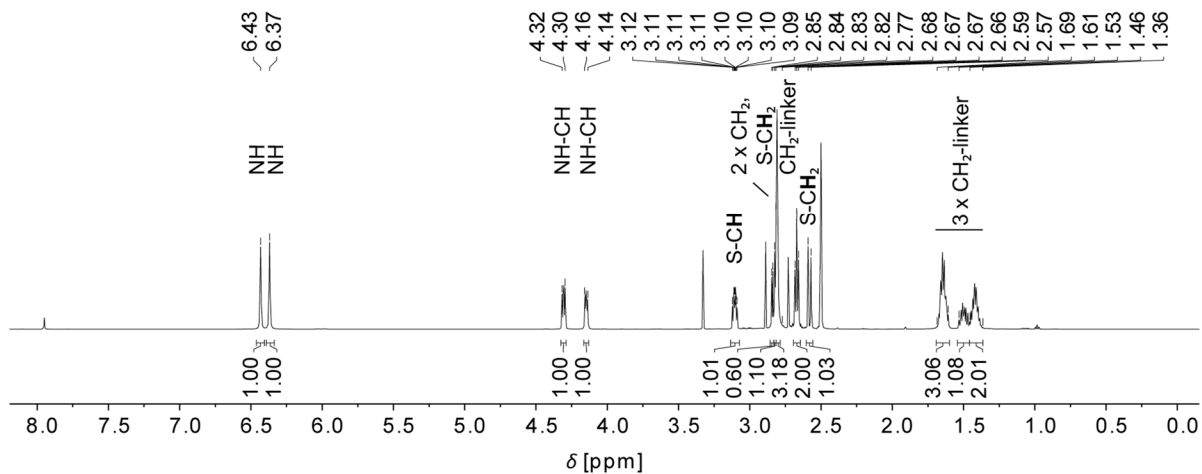
**<sup>1</sup>H NMR** (MeOD, 400 MHz)**<sup>13</sup>C NMR** (MeOD, 100 MHz)

**<sup>1</sup>H NMR** (MeOD, 400 MHz)**<sup>13</sup>C NMR** (DMSO-*d*<sub>6</sub>, 100 MHz)**<sup>19</sup>F NMR** (DMSO-*d*<sub>6</sub>, 376 MHz)

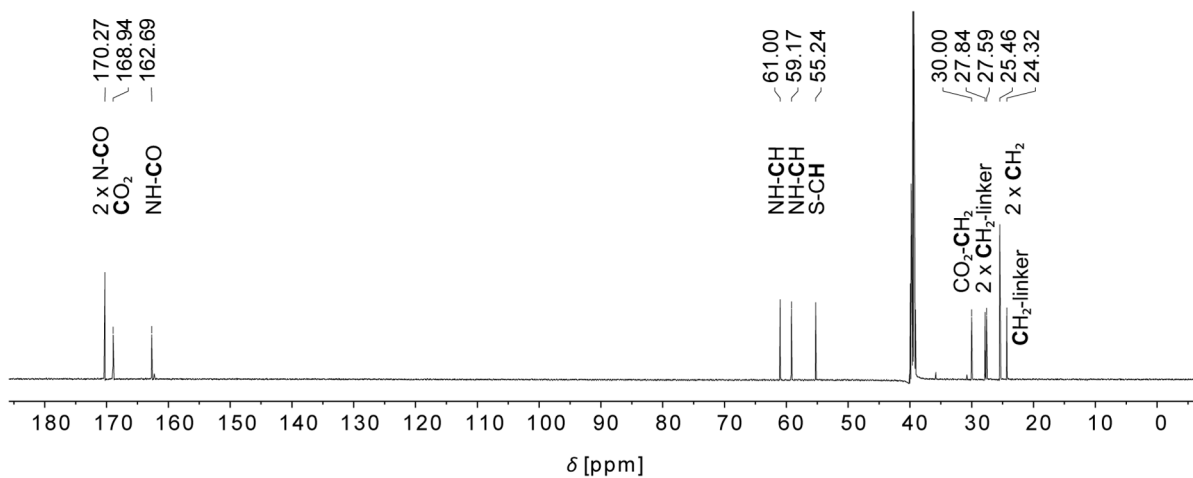
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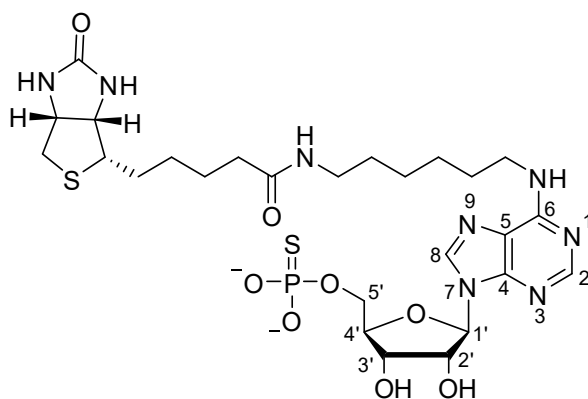


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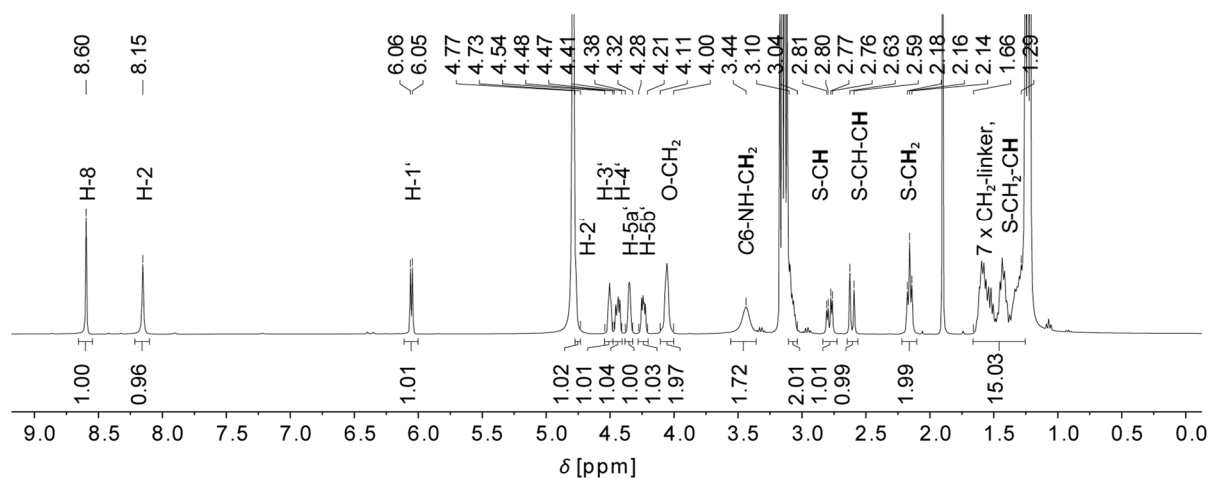


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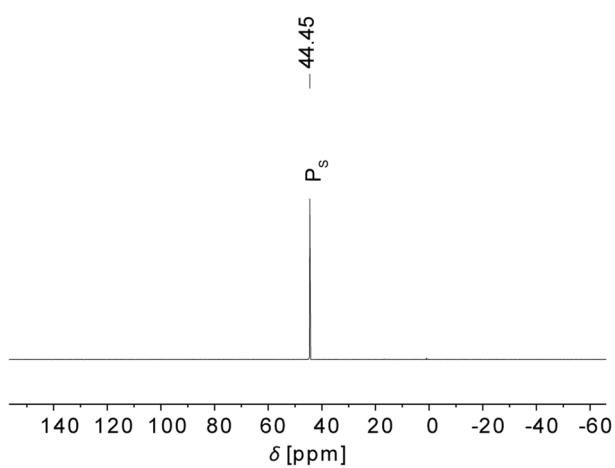


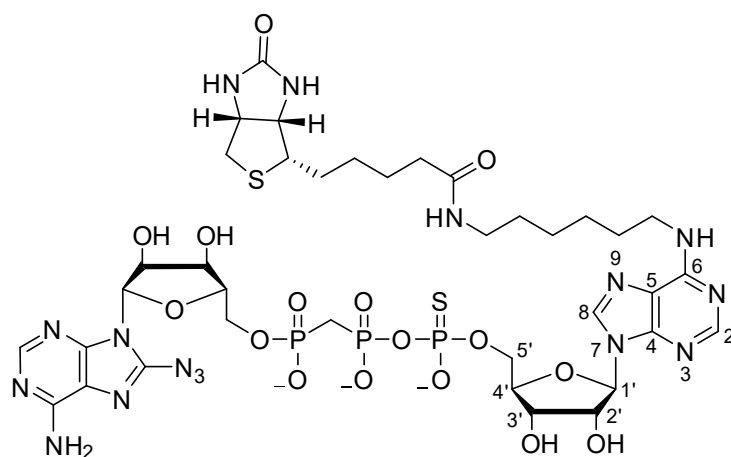
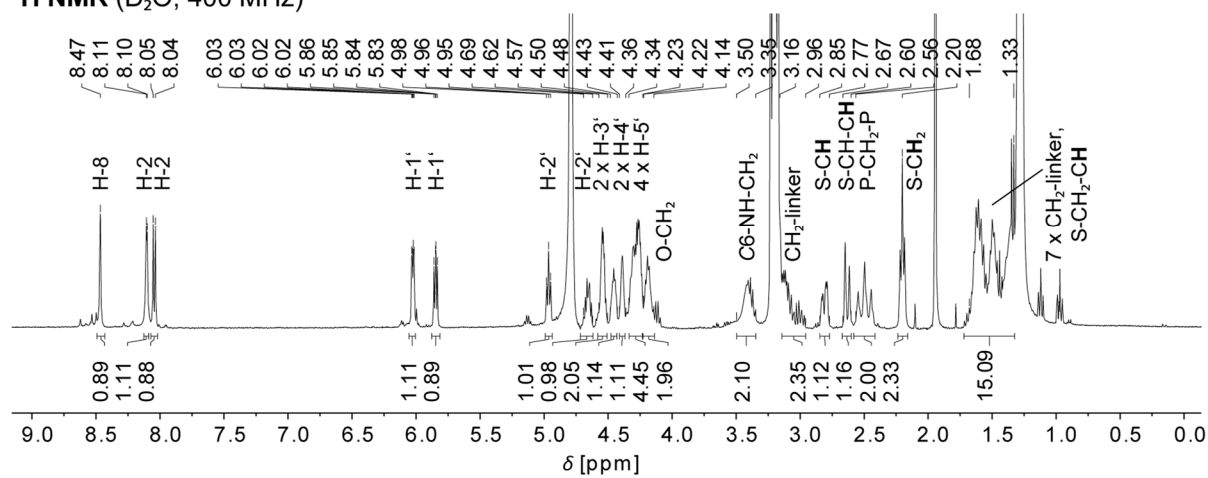
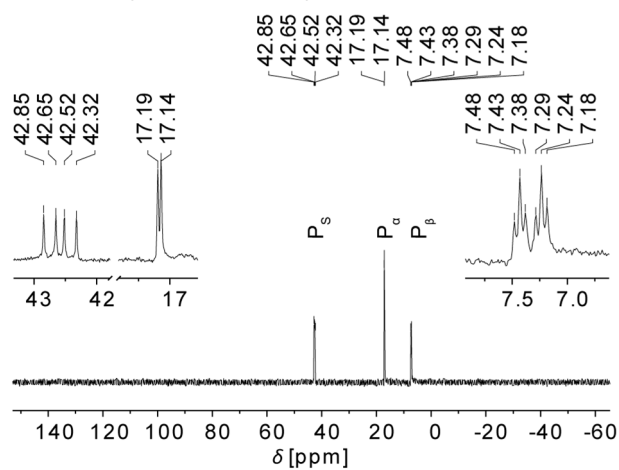


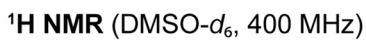
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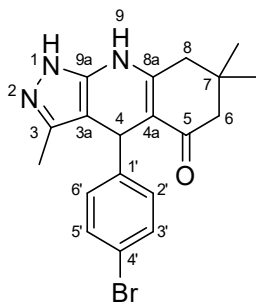


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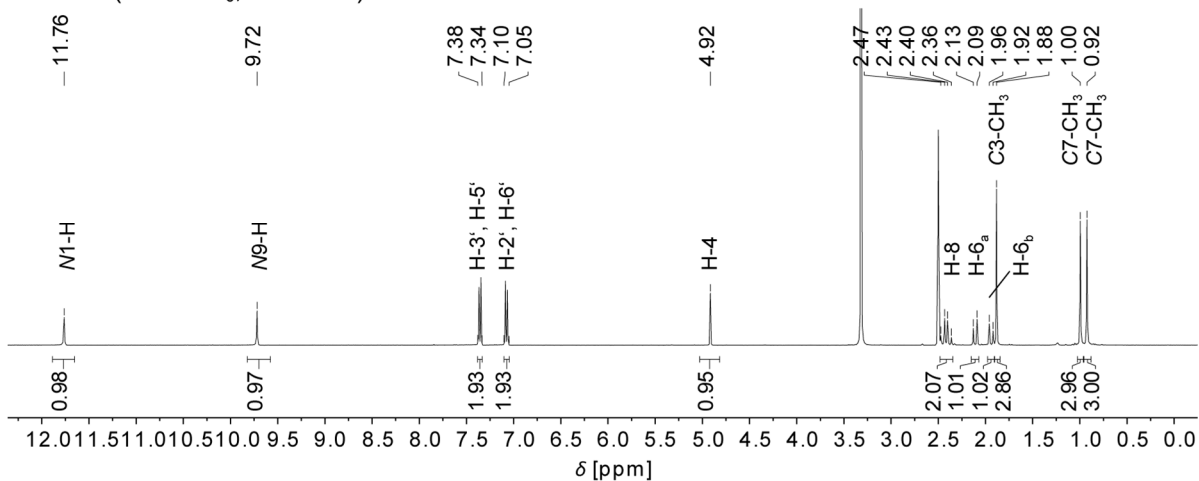


 $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz) $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , 161 MHz)

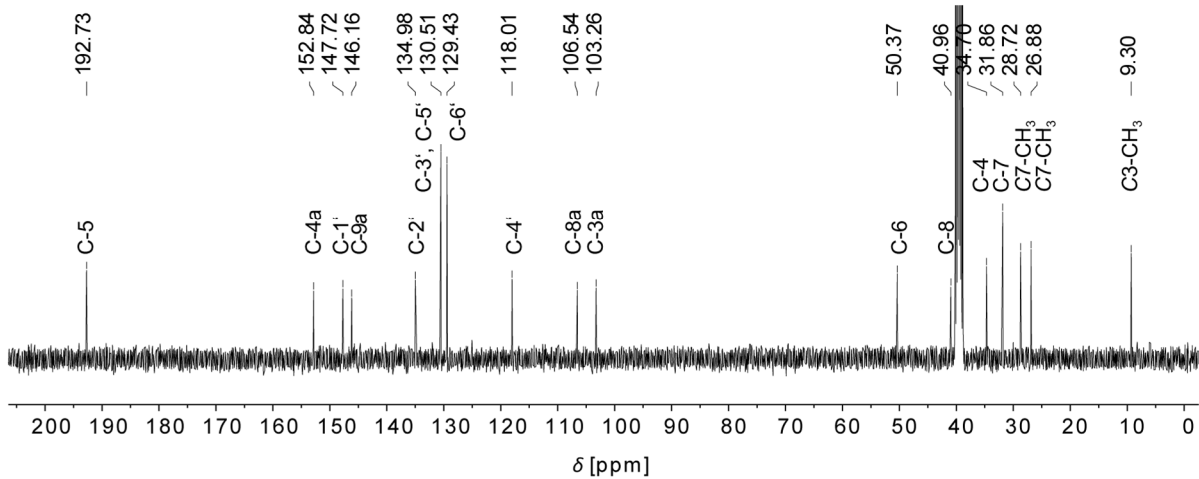


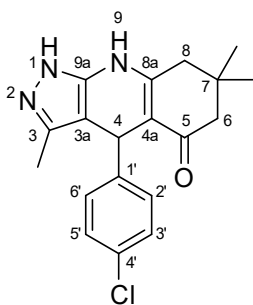
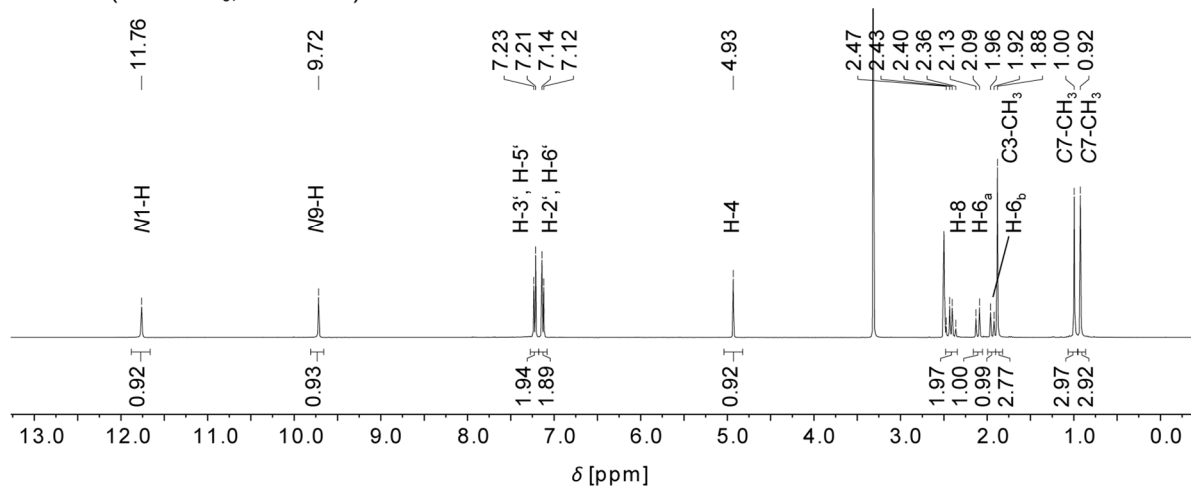
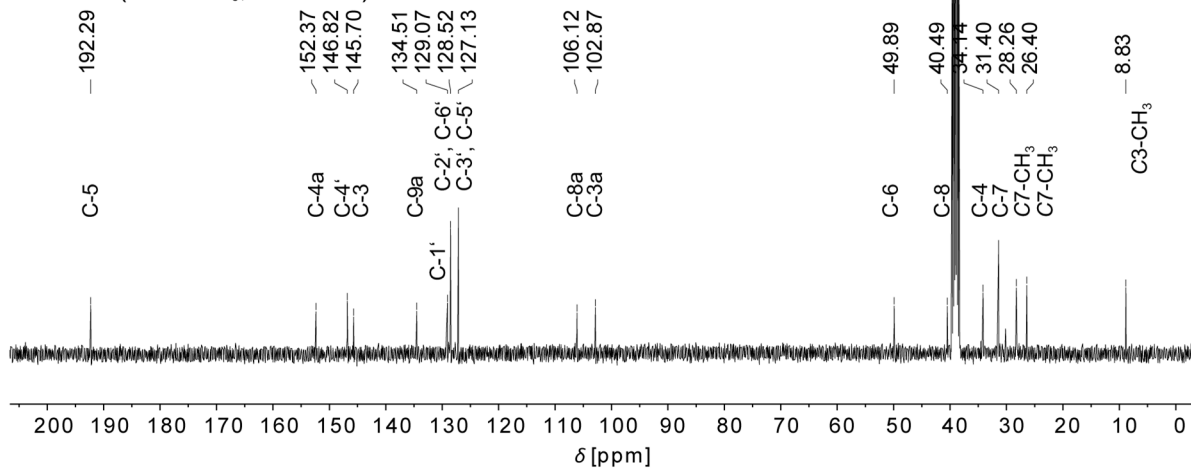


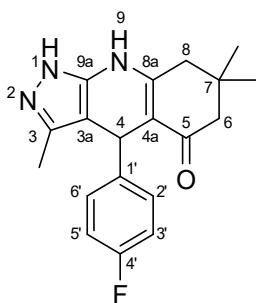
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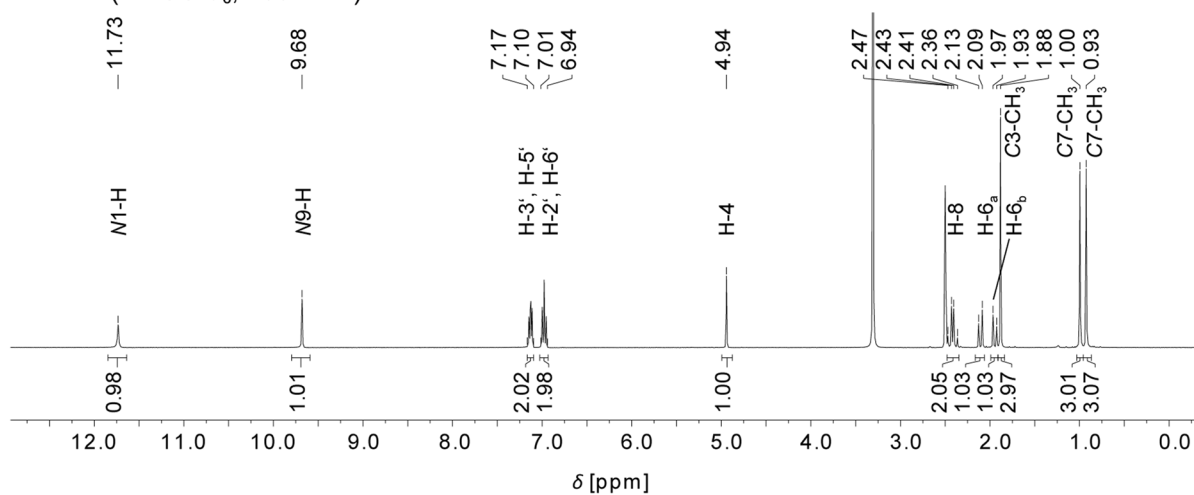
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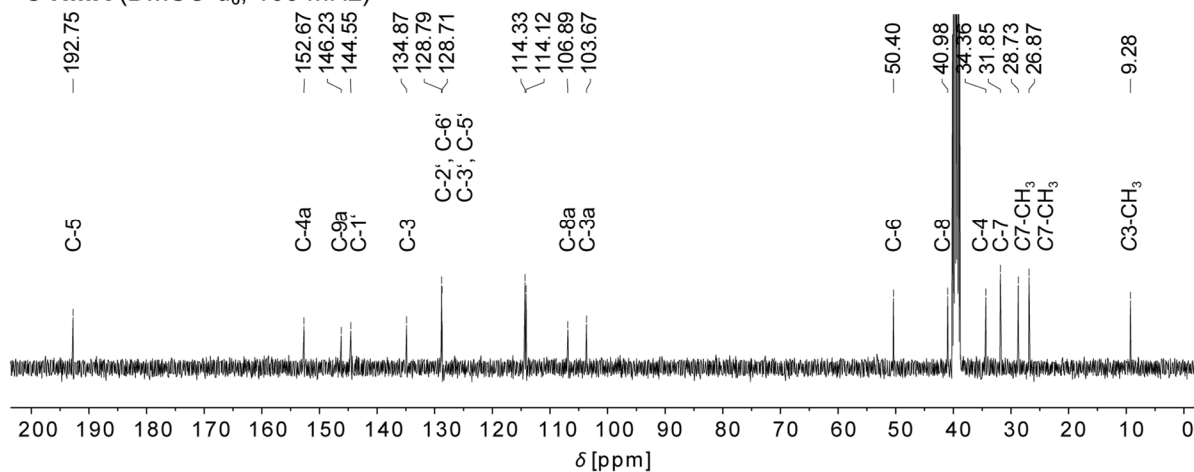
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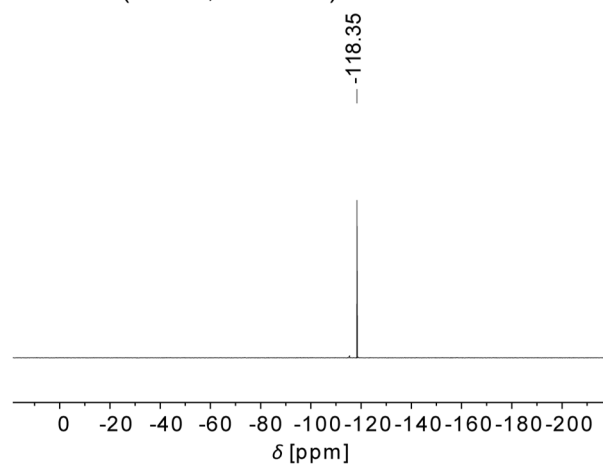
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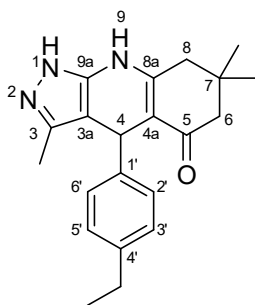
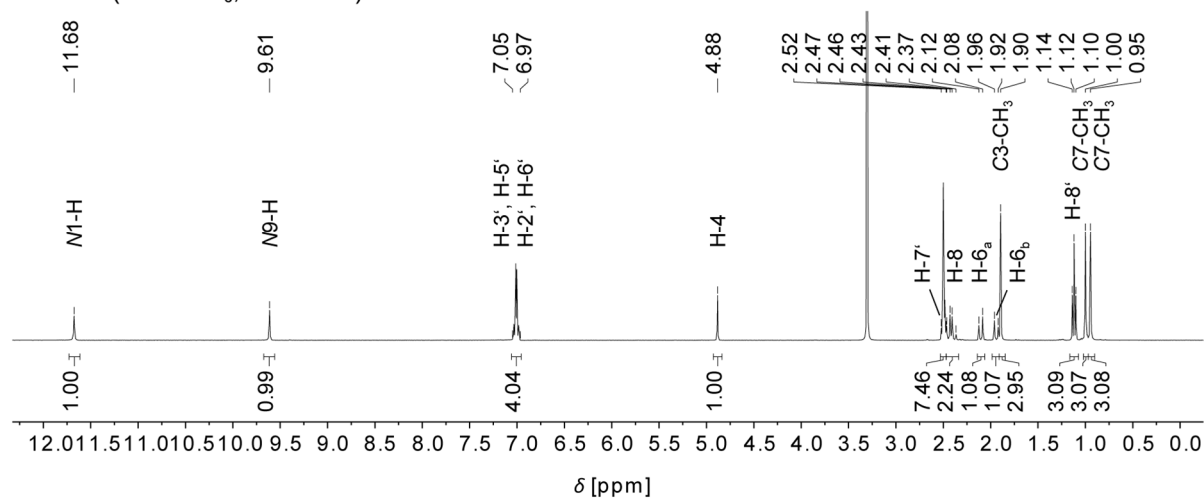
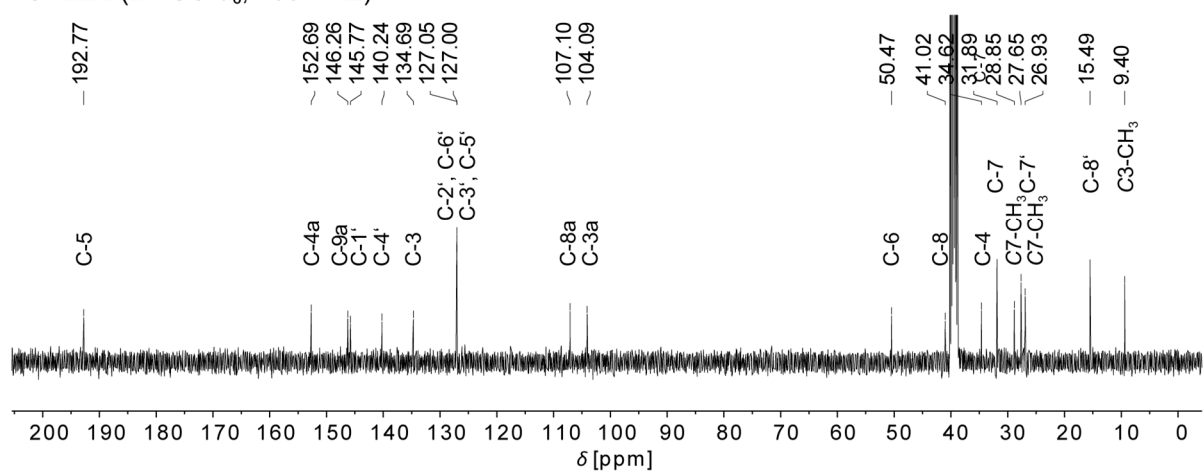


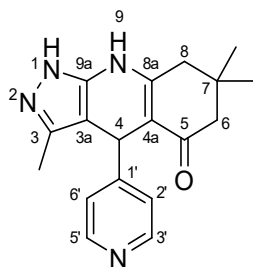
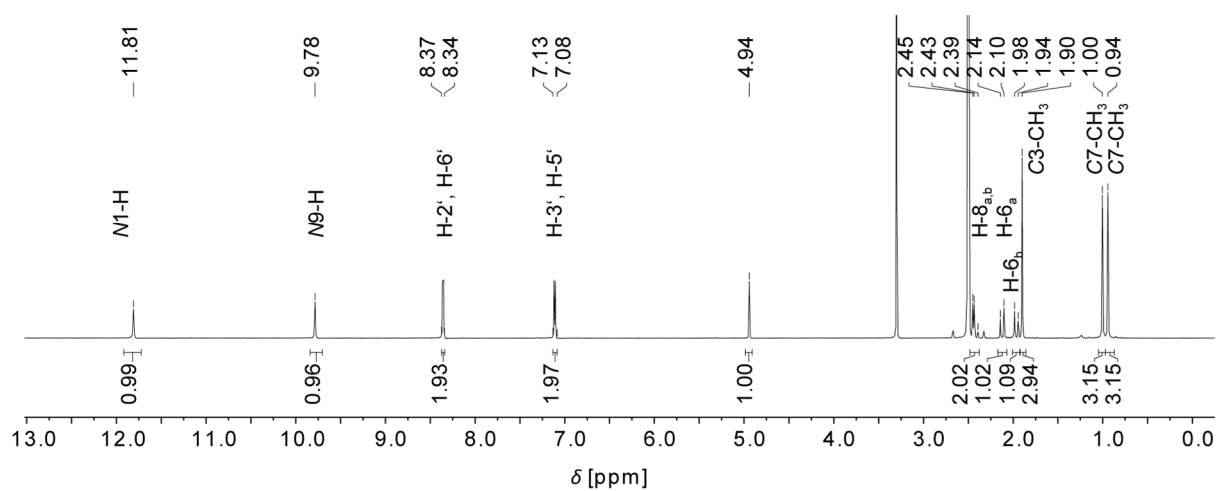
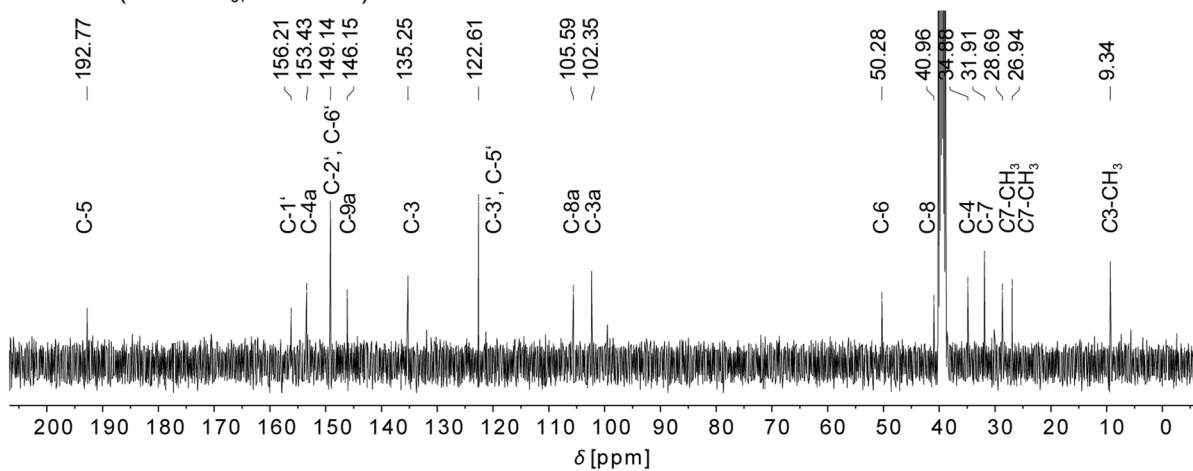
$^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz)

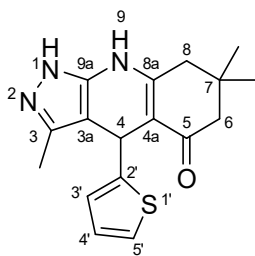
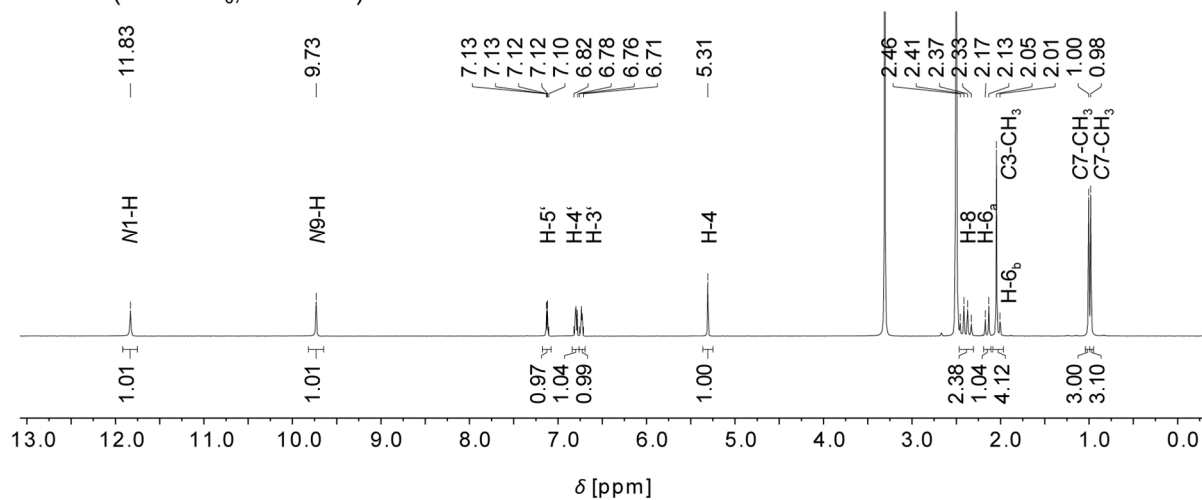
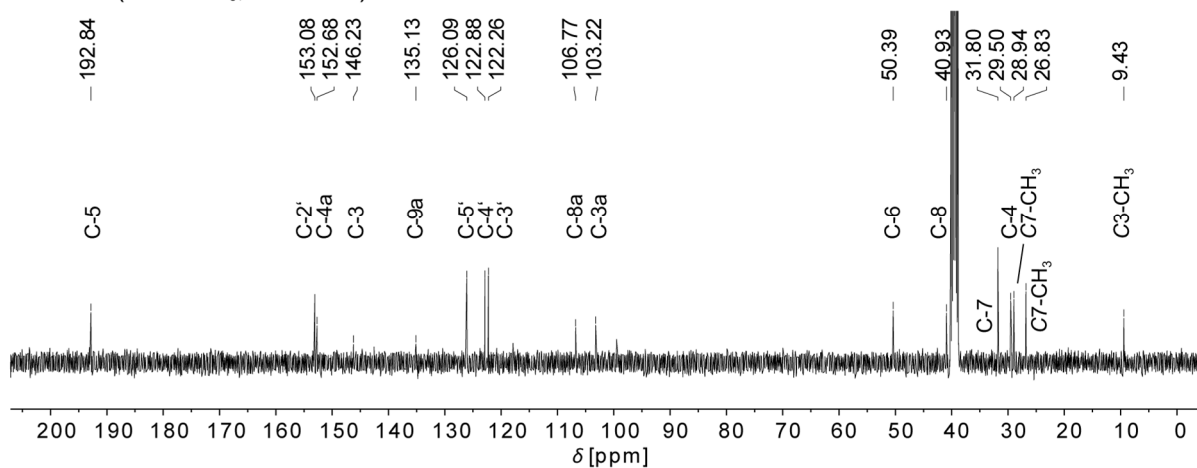


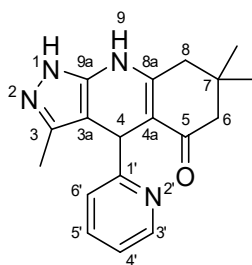
$^{19}\text{F}$  NMR (MeOD, 376 MHz)



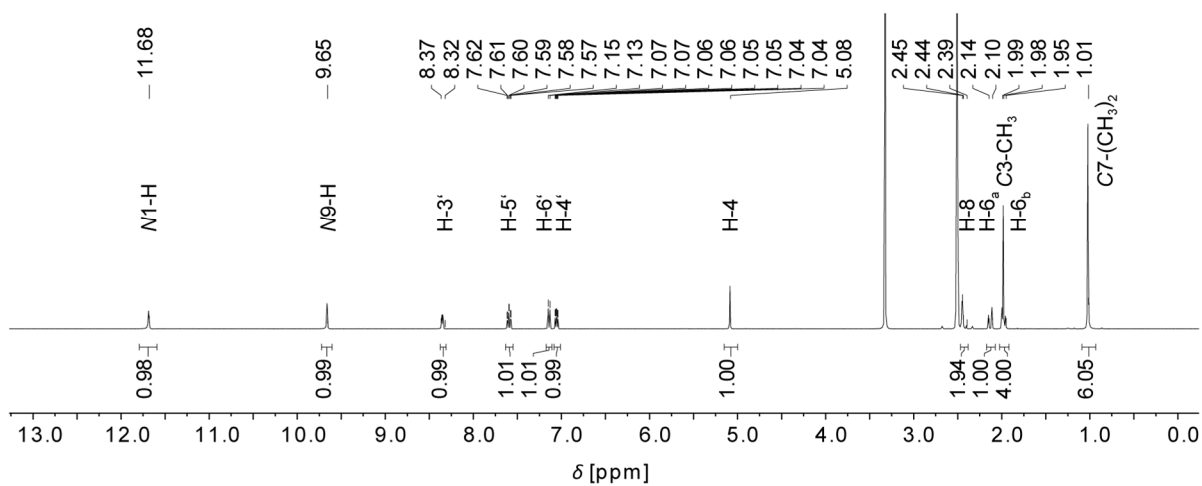
**<sup>1</sup>H NMR** (DMSO-*d*<sub>6</sub>, 400 MHz)**<sup>13</sup>C NMR** (DMSO-*d*<sub>6</sub>, 100 MHz)

**<sup>1</sup>H NMR** (DMSO-*d*<sub>6</sub>, 400 MHz)**<sup>13</sup>C NMR** (DMSO-*d*<sub>6</sub>, 100 MHz)

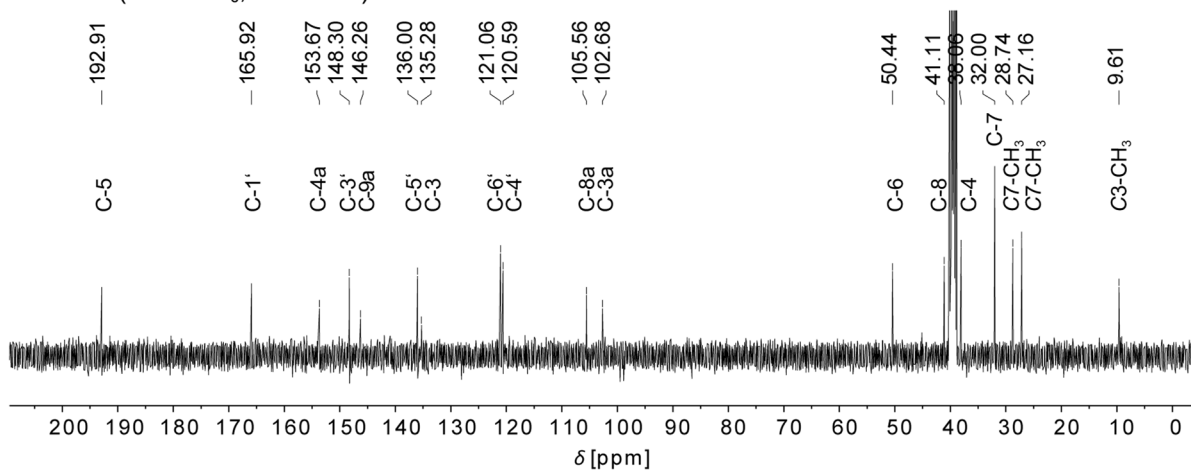
**<sup>1</sup>H NMR** (DMSO-*d*<sub>6</sub>, 400 MHz)**<sup>13</sup>C NMR** (DMSO-*d*<sub>6</sub>, 100 MHz)

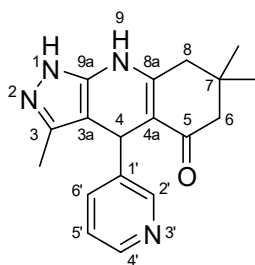
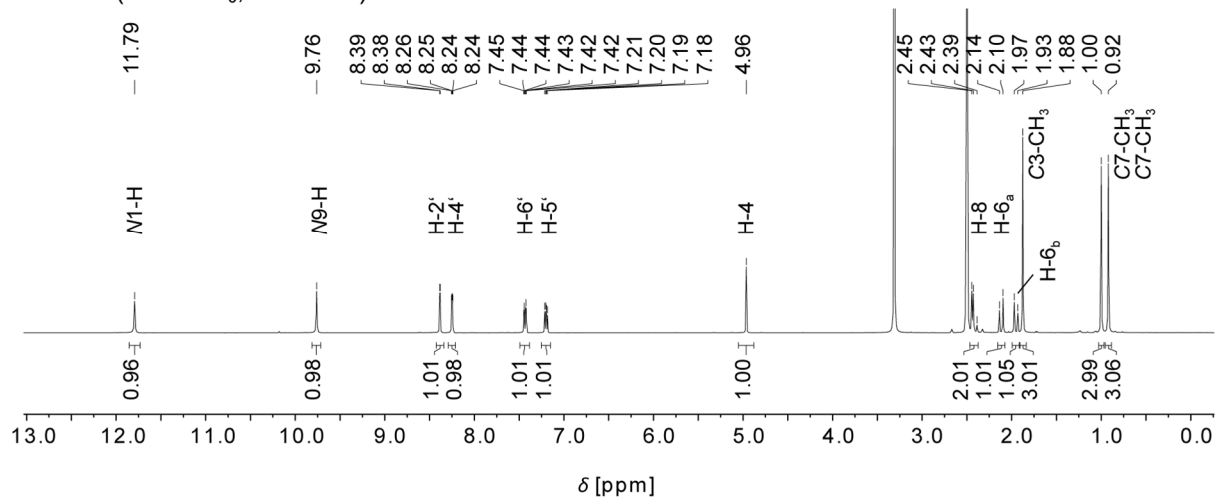
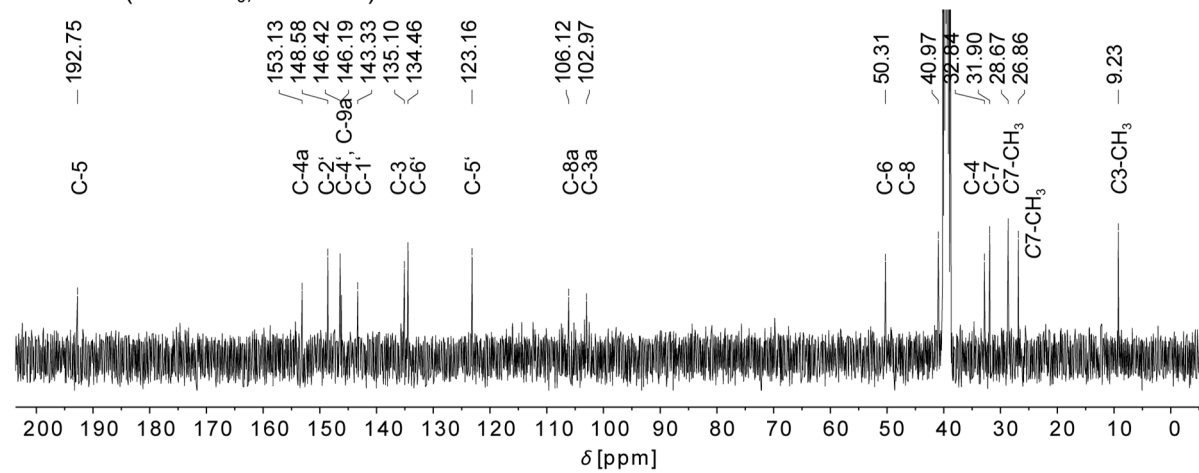


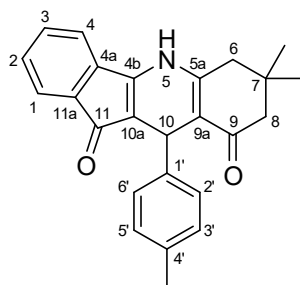
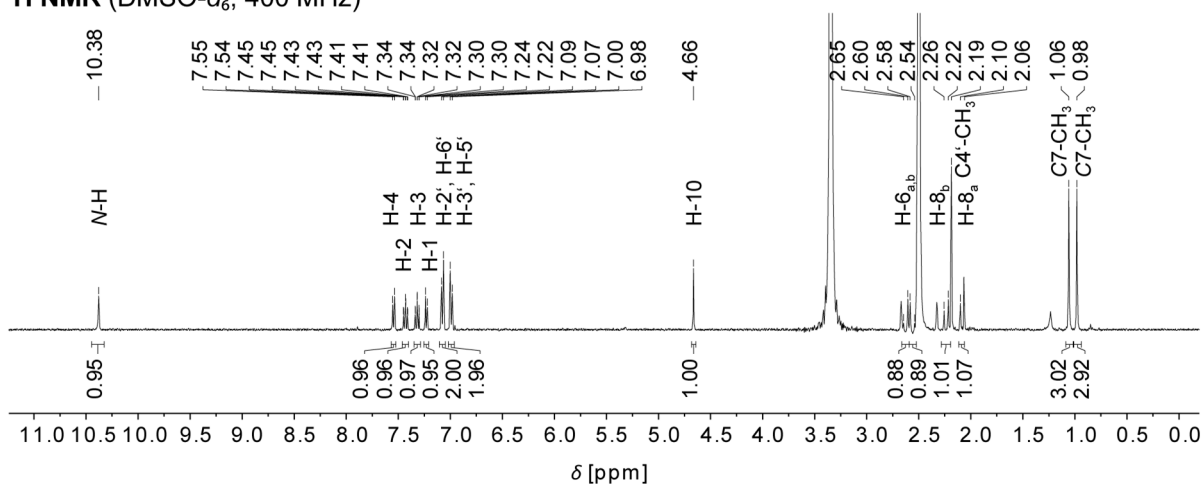
$^1\text{H}$  NMR (DMSO- $d_6$ , 400 MHz)



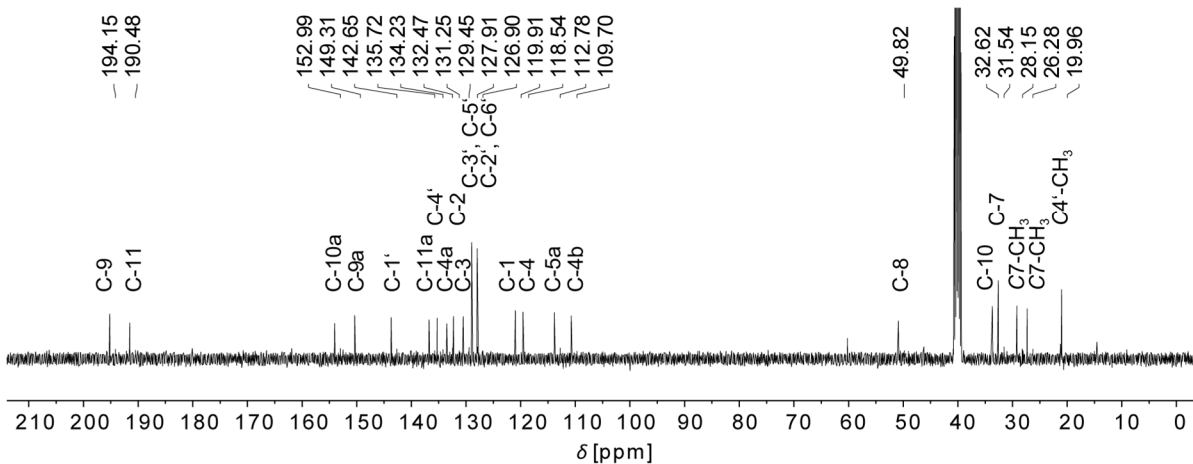
$^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz)



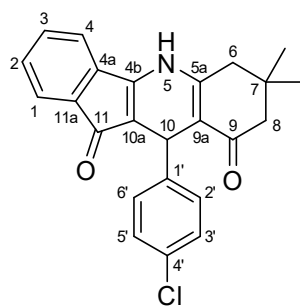
**<sup>1</sup>H NMR** (DMSO-*d*<sub>6</sub>, 400 MHz)**<sup>13</sup>C NMR** (DMSO-*d*<sub>6</sub>, 100 MHz)

<sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz)

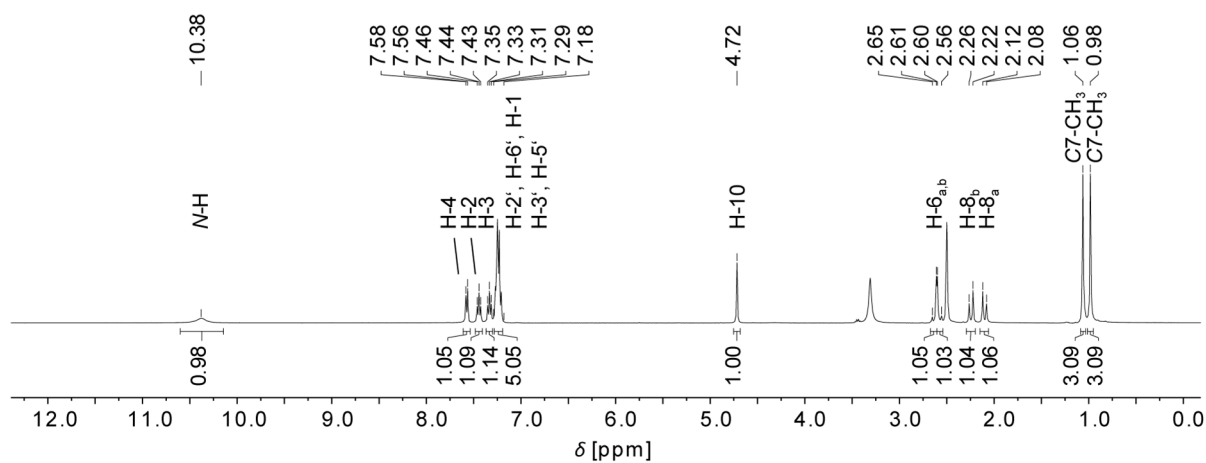
**$^{13}\text{C}$  NMR** (DMSO- $d_6$ , 100 MHz)



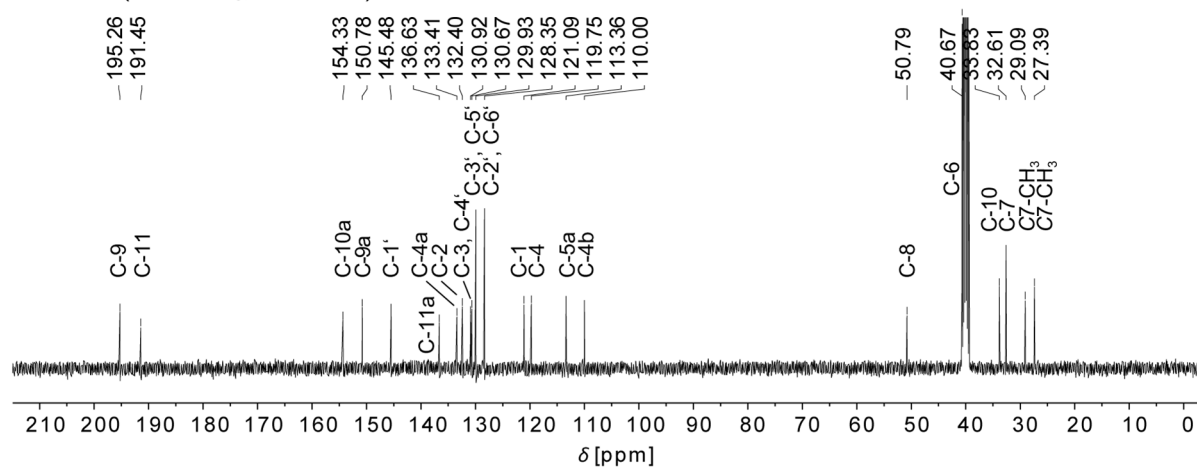


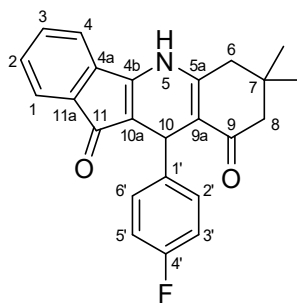
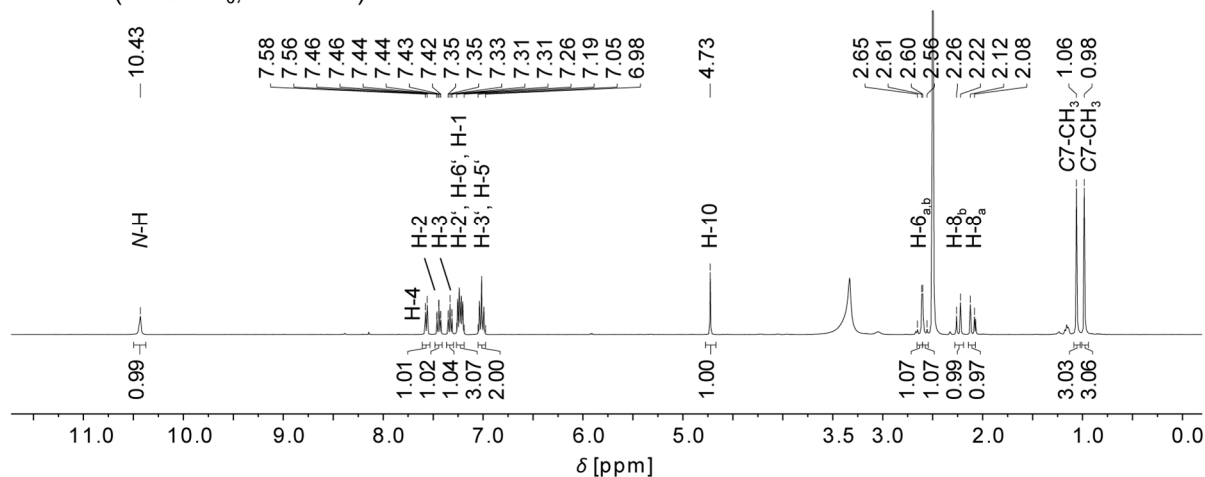
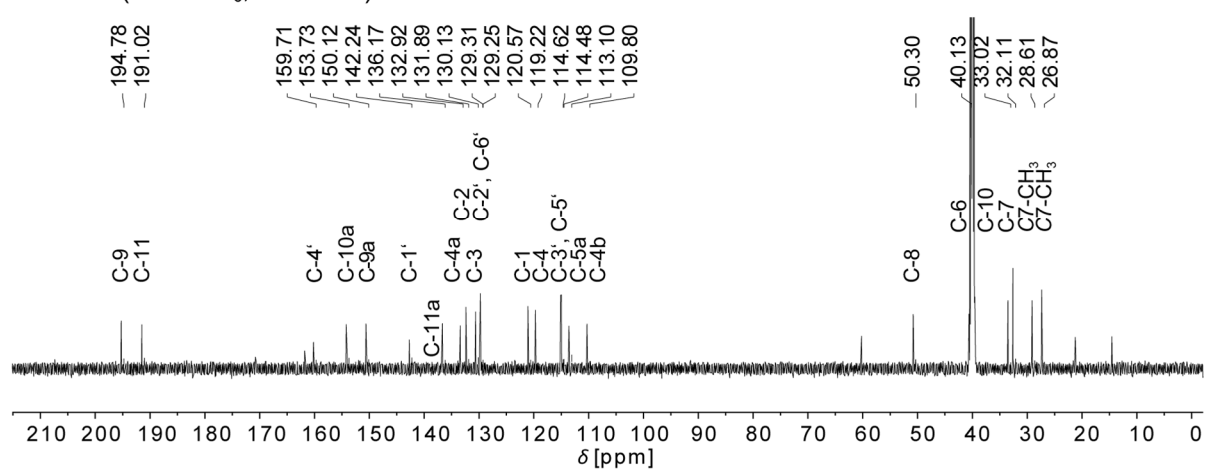
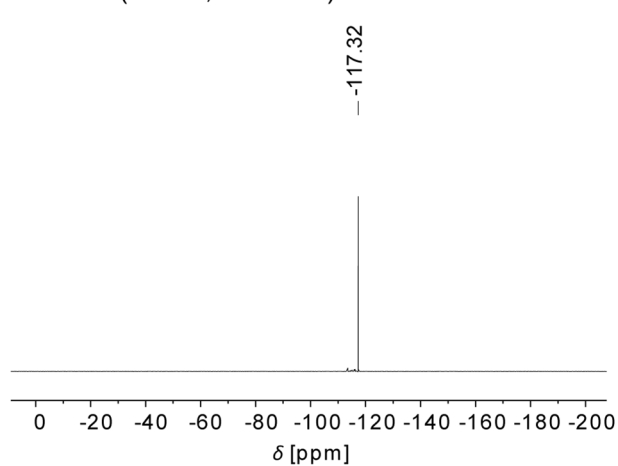


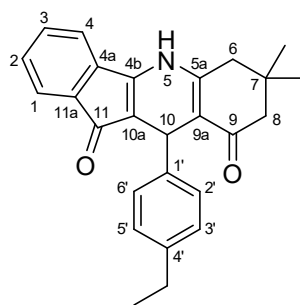
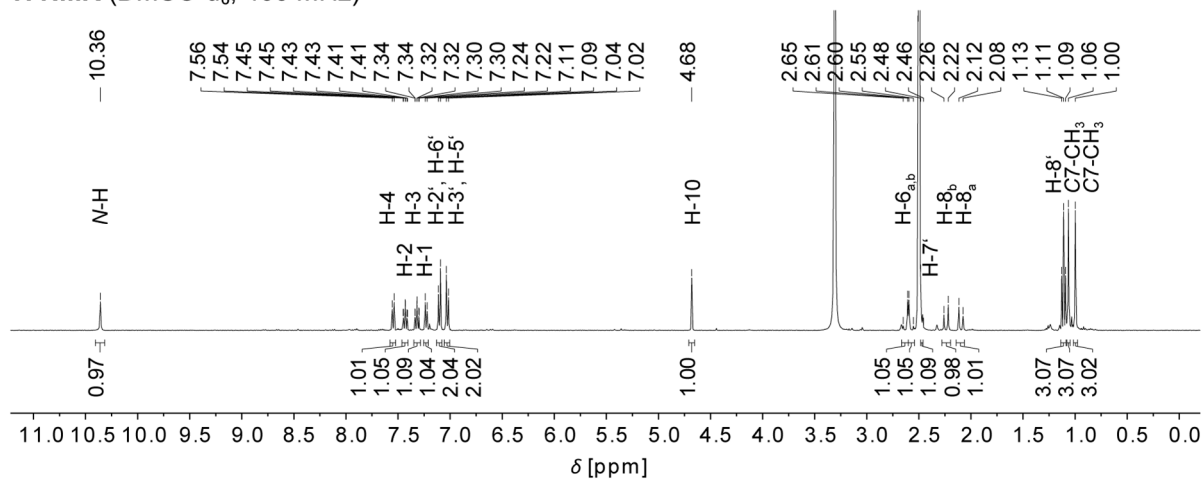
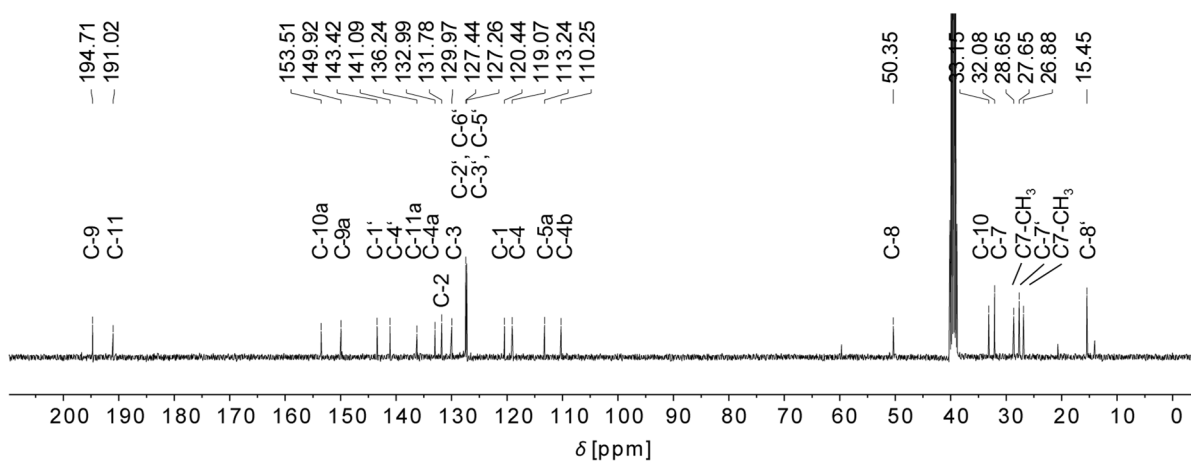
$^1\text{H}$  NMR (DMSO- $d_6$ , 400 MHz)

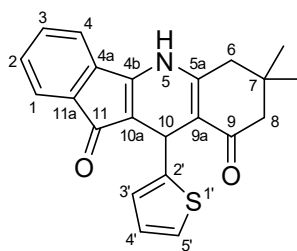
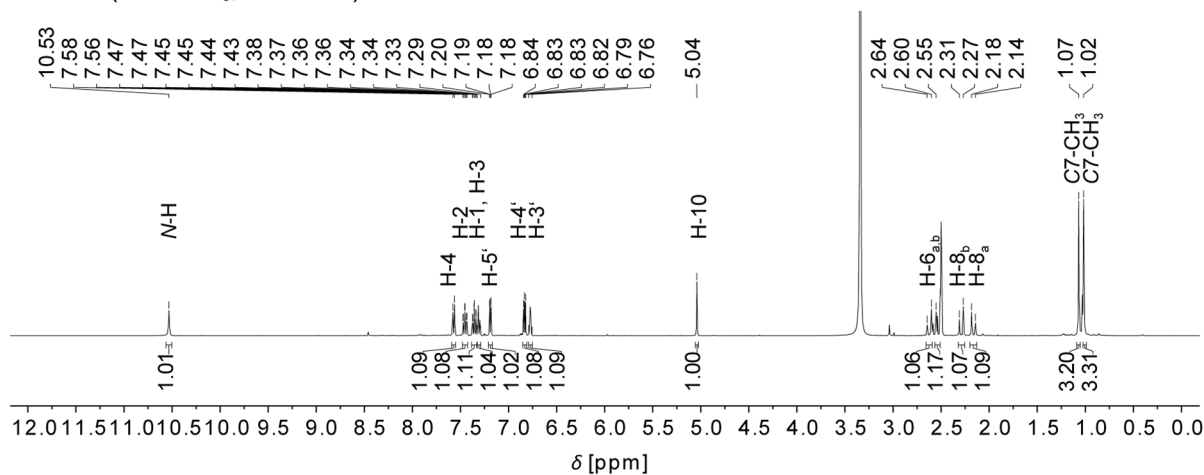
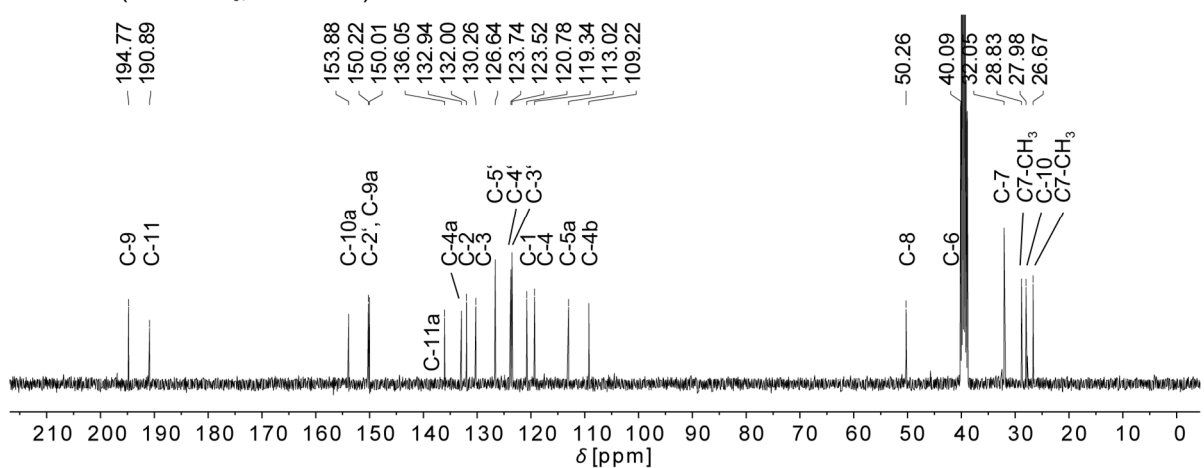


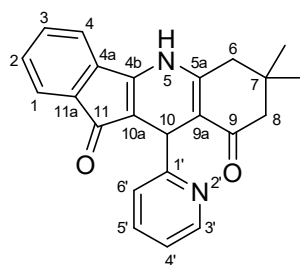
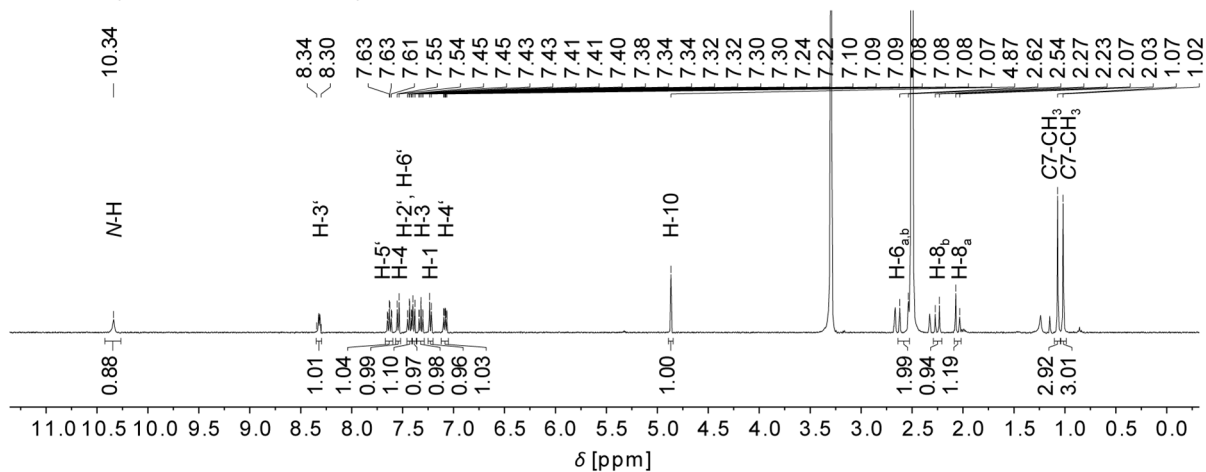
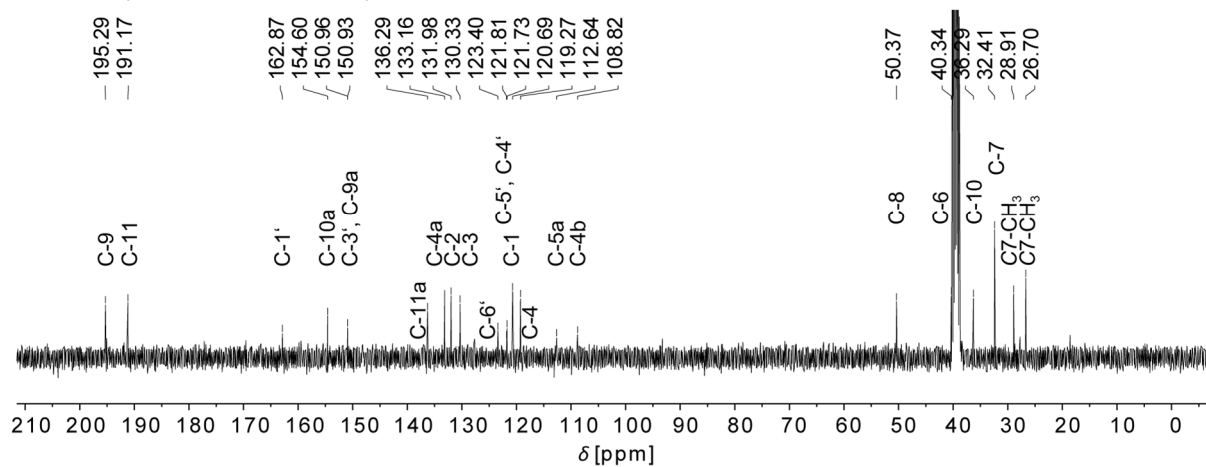
$^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz)

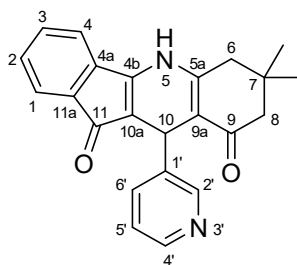


 $^1\text{H}$  NMR (DMSO- $d_6$ , 400 MHz) $^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz) $^{19}\text{F}$  NMR (MeOD, 376 MHz)

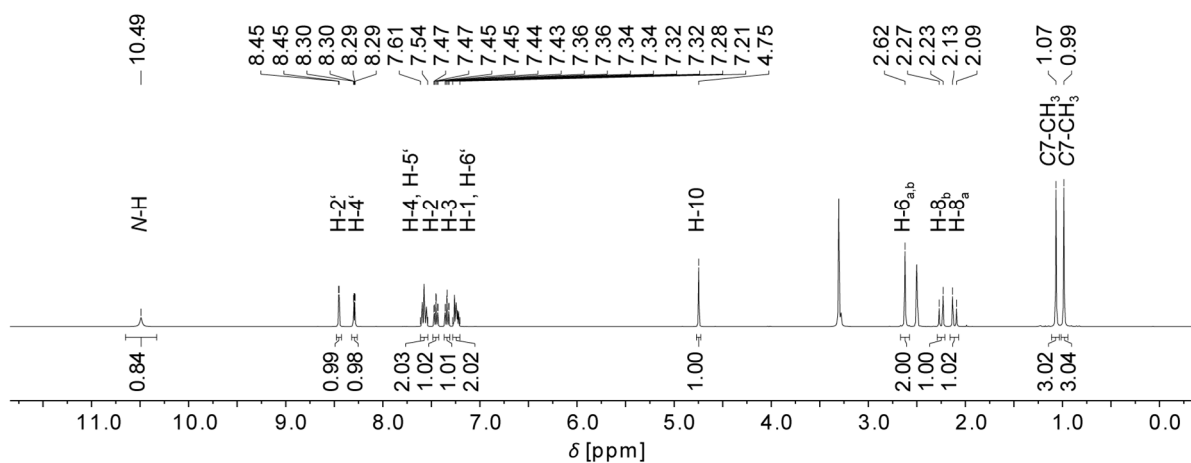
**<sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz)****<sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz)**

**<sup>1</sup>H NMR** (DMSO-*d*<sub>6</sub>, 400 MHz)**<sup>13</sup>C NMR** (DMSO-*d*<sub>6</sub>, 100 MHz)

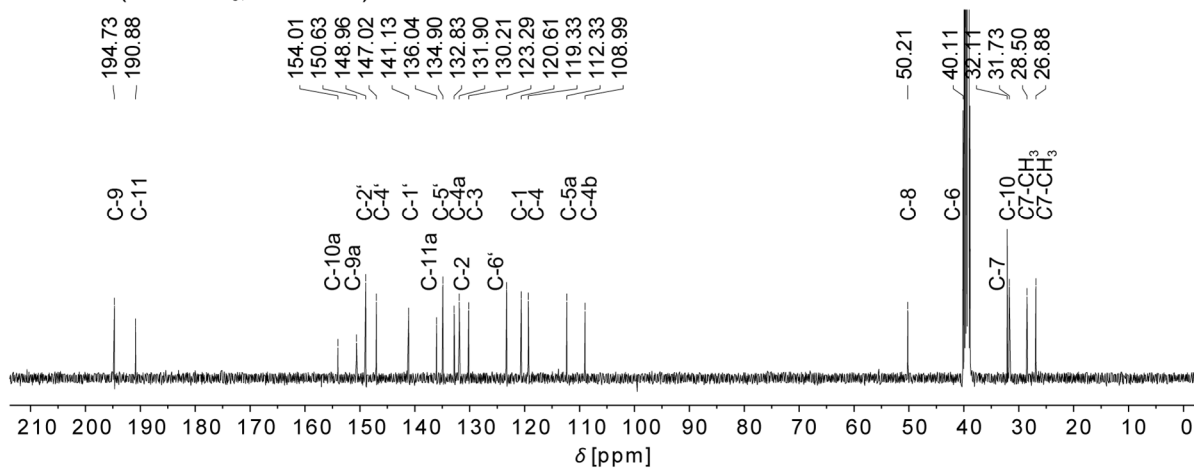
 **$^1\text{H}$  NMR (DMSO- $d_6$ , 400 MHz)** **$^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz)**

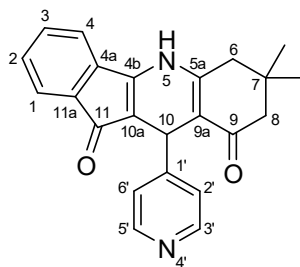
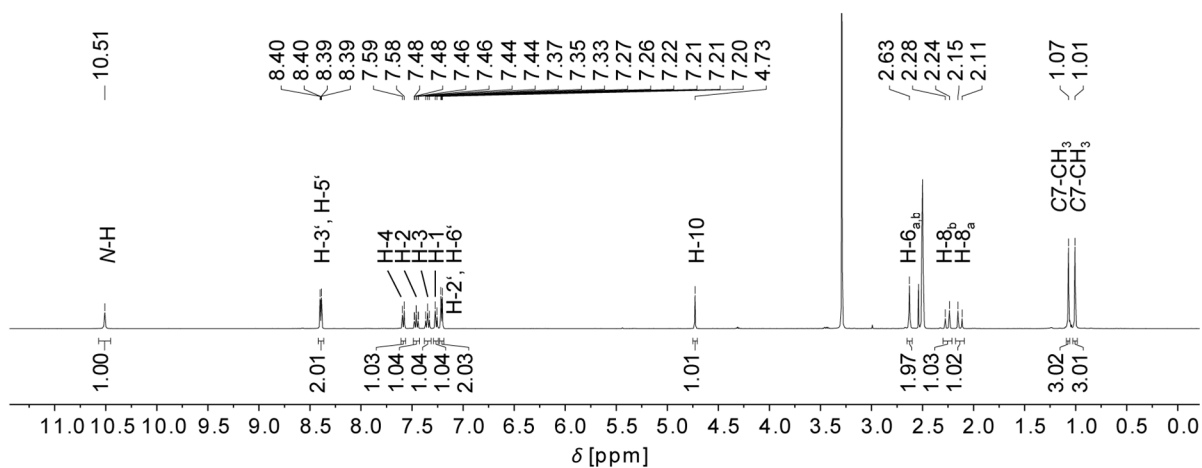
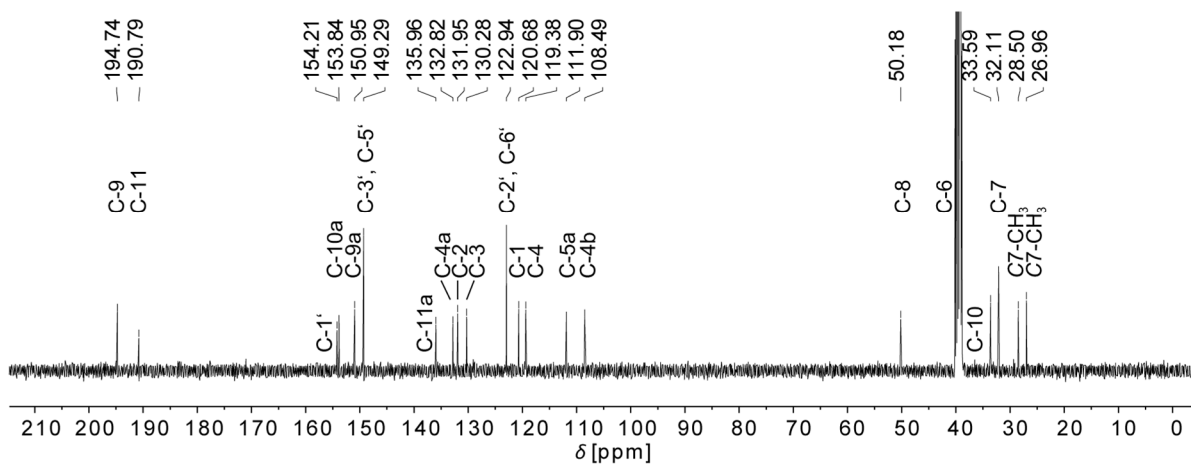


$^1\text{H}$  NMR (DMSO- $d_6$ , 400 MHz)



$^{13}\text{C}$  NMR (DMSO- $d_6$ , 100 MHz)



**<sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>, 400 MHz)****<sup>13</sup>C NMR (DMSO-*d*<sub>6</sub>, 100 MHz)**

## 2 Proteins Identified from the ABPP Experiments

### 2.1 Proteins Significantly Enriched in the ABPP with Probe C in Comparison to the Beads Control

**Table 7:** Proteins significantly enriched in the ABPP with probe C in comparison to the beads control.

|    | Significant | -log p | Difference | Majority protein IDs | Protein names                                                                                                                  | Gene names |
|----|-------------|--------|------------|----------------------|--------------------------------------------------------------------------------------------------------------------------------|------------|
| 1  | +           | 11.932 | 5.056      | P04406               | Glyceraldehyde-3-phosphate dehydrogenase                                                                                       | GAPDH      |
| 2  | +           | 6.272  | 4.894      | P34932               | Heat shock 70 kDa protein 4                                                                                                    | HSPA4      |
| 3  | +           | 4.656  | 4.624      | Q9HB71               | Calcyclin-binding protein                                                                                                      | CACYBP     |
| 4  | +           | 9.639  | 4.622      | P27348               | 14-3-3 protein theta                                                                                                           | YWHAQ      |
| 5  | +           | 3.531  | 4.533      | P37802               | Transgelin-2                                                                                                                   | TAGLN2     |
| 6  | +           | 5.573  | 4.443      | Q9Y490               | Talin-1                                                                                                                        | TLN1       |
| 7  | +           | 5.851  | 4.425      | Q92598               | Heat shock protein 105 kDa                                                                                                     | HSPH1      |
| 8  | +           | 6.646  | 4.373      | P21333               | Filamin-A                                                                                                                      | FLNA       |
| 9  | +           | 4.527  | 4.302      | P12268               | Inosine-5-monophosphate dehydrogenase 2                                                                                        | IMPDH2     |
| 10 | +           | 3.524  | 4.301      | P30086               | Phosphatidylethanolamine-binding protein 1;Hippocampal cholinergic neurostimulating peptide                                    | PEBP1      |
| 11 | +           | 4.780  | 4.279      | P13797               | Plastin-3                                                                                                                      | PLS3       |
| 12 | +           | 5.300  | 4.185      | P40926               | Malate dehydrogenase, mitochondrial                                                                                            | MDH2       |
| 13 | +           | 4.789  | 4.178      | P54136               | Arginine--tRNA ligase, cytoplasmic                                                                                             | RARS       |
| 14 | +           | 4.511  | 4.161      | P07900               | Heat shock protein HSP 90-alpha                                                                                                | HSP90AA1   |
| 15 | +           | 8.246  | 4.154      | Q9P2J5               | Leucine--tRNA ligase, cytoplasmic                                                                                              | LARS       |
| 16 | +           | 4.001  | 4.108      | P22234               | Multifunctional protein ADE2;Phosphoribosylaminoimidazole-succinocarboxamide synthase;Phosphoribosylaminoimidazole carboxylase | PAICS      |
| 17 | +           | 2.886  | 4.108      | Q13162               | Peroxioredoxin-4                                                                                                               | PRDX4      |
| 18 | +           | 5.547  | 4.011      | P35579               | Myosin-9                                                                                                                       | MYH9       |
| 19 | +           | 3.822  | 3.998      | P14625               | Endoplasmic                                                                                                                    | HSP90B1    |
| 20 | +           | 10.694 | 3.995      | P33993               | DNA replication licensing factor MCM7                                                                                          | MCM7       |

## Appendix

|    |   |       |       |        |                                                                                                             |           |
|----|---|-------|-------|--------|-------------------------------------------------------------------------------------------------------------|-----------|
| 21 | + | 3.799 | 3.980 | P08865 | 40S ribosomal protein SA                                                                                    | RPSA      |
| 22 | + | 6.925 | 3.942 | P61221 | ATP-binding cassette sub-family E member 1                                                                  | ABCE1     |
| 23 | + | 3.580 | 3.932 | Q02790 | Peptidyl-prolyl cis-trans isomerase FKBP4;Peptidyl-prolyl cis-trans isomerase FKBP4, N-terminally processed | FKBP4     |
| 24 | + | 7.356 | 3.910 | Q15181 | Inorganic pyrophosphatase                                                                                   | PPA1      |
| 25 | + | 4.022 | 3.842 | P22314 | Ubiquitin-like modifier-activating enzyme 1                                                                 | UBA1      |
| 26 | + | 6.892 | 3.823 | Q9UQ80 | Proliferation-associated protein 2G4                                                                        | PA2G4     |
| 27 | + | 5.150 | 3.822 | P25205 | DNA replication licensing factor MCM3                                                                       | MCM3      |
| 28 | + | 5.713 | 3.806 | P26641 | Elongation factor 1-gamma                                                                                   | EEF1G     |
| 29 | + | 3.540 | 3.800 | P22626 | Heterogeneous nuclear ribonucleoproteins A2/B1                                                              | HNRNPA2B1 |
| 30 | + | 3.790 | 3.791 | P31948 | Stress-induced-phosphoprotein 1                                                                             | STIP1     |
| 31 | + | 5.625 | 3.785 | P60891 | Ribose-phosphate pyrophosphokinase 1                                                                        | PRPS1     |
| 32 | + | 8.374 | 3.770 | Q14166 | Tubulin--tyrosine ligase-like protein 12                                                                    | TTL12     |
| 33 | + | 8.442 | 3.766 | O43390 | Heterogeneous nuclear ribonucleoprotein R                                                                   | HNRNPR    |
| 34 | + | 4.918 | 3.764 | P53396 | ATP-citrate synthase                                                                                        | ACLY      |
| 35 | + | 8.224 | 3.724 | P56192 | Methionine--tRNA ligase, cytoplasmic                                                                        | MARS      |
| 36 | + | 4.335 | 3.679 | Q58FF8 | Putative heat shock protein HSP 90-beta 2                                                                   | HSP90AB2P |
| 37 | + | 4.777 | 3.642 | P49736 | DNA replication licensing factor MCM2                                                                       | MCM2      |
| 38 | + | 5.686 | 3.634 | P49588 | Alanine--tRNA ligase, cytoplasmic                                                                           | AARS      |
| 39 | + | 3.828 | 3.623 | P07237 | Protein disulfide-isomerase                                                                                 | P4HB      |
| 40 | + | 7.849 | 3.585 | P62136 | Serine/threonine-protein phosphatase PP1-alpha catalytic subunit                                            | PPP1CA    |
| 41 | + | 4.109 | 3.542 | P18669 | Phosphoglycerate mutase 1                                                                                   | PGAM1     |
| 42 | + | 7.313 | 3.539 | P07814 | Bifunctional glutamate/proline--tRNA ligase;Glutamate--tRNA ligase;Proline--tRNA ligase                     | EPRS      |
| 43 | + | 5.303 | 3.535 | P39748 | Flap endonuclease 1                                                                                         | FEN1      |
| 44 | + | 5.710 | 3.514 | Q14566 | DNA replication licensing factor MCM6                                                                       | MCM6      |
| 45 | + | 6.427 | 3.489 | P41252 | Isoleucine--tRNA ligase, cytoplasmic                                                                        | IARS      |
| 46 | + | 1.965 | 3.477 | P12277 | Creatine kinase B-type                                                                                      | CKB       |
| 47 | + | 3.148 | 3.471 | P30101 | Protein disulfide-isomerase A3                                                                              | PDI3      |
| 48 | + | 6.683 | 3.460 | P38919 | Eukaryotic initiation factor 4A-III;Eukaryotic initiation factor 4A-III, N-terminally processed             | EIF4A3    |

## Identified Proteins

|    |   |       |       |               |                                                                                               |               |
|----|---|-------|-------|---------------|-----------------------------------------------------------------------------------------------|---------------|
| 49 | + | 4.989 | 3.459 | P54577        | Tyrosine--tRNA ligase, cytoplasmic;Tyrosine--tRNA ligase, cytoplasmic, N-terminally processed | YARS          |
| 50 | + | 3.792 | 3.446 | Q14103        | Heterogeneous nuclear ribonucleoprotein D0                                                    | HNRNPD        |
| 51 | + | 3.320 | 3.440 | P00338        | L-lactate dehydrogenase A chain                                                               | LDHA          |
| 52 | + | 5.577 | 3.437 | P26640        | Valine--tRNA ligase                                                                           | VAR5          |
| 53 | + | 4.106 | 3.414 | P23284        | Peptidyl-prolyl cis-trans isomerase B                                                         | PIIB          |
| 54 | + | 4.371 | 3.392 | P62837;P61077 | Ubiquitin-conjugating enzyme E2 D2;Ubiquitin-conjugating enzyme E2 D3                         | UBE2D2;UBE2D3 |
| 55 | + | 4.600 | 3.389 | Q9Y265        | RuvB-like 1                                                                                   | RUVBL1        |
| 56 | + | 3.838 | 3.364 | P06733        | Alpha-enolase                                                                                 | ENO1          |
| 57 | + | 6.546 | 3.338 | P18754        | Regulator of chromosome condensation                                                          | RCC1          |
| 58 | + | 2.574 | 3.328 | P04075        | Fructose-bisphosphate aldolase A                                                              | ALDOA         |
| 59 | + | 3.214 | 3.328 | P30048        | Thioredoxin-dependent peroxide reductase, mitochondrial                                       | PRDX3         |
| 60 | + | 6.945 | 3.315 | P62495        | Eukaryotic peptide chain release factor subunit 1                                             | ETF1          |
| 61 | + | 3.117 | 3.313 | Q12906        | Interleukin enhancer-binding factor 3                                                         | ILF3          |
| 62 | + | 8.374 | 3.311 | P18206        | Vinculin                                                                                      | VCL           |
| 63 | + | 4.190 | 3.288 | P06493        | Cyclin-dependent kinase 1                                                                     | CDK1          |
| 64 | + | 4.141 | 3.270 | P33991        | DNA replication licensing factor MCM4                                                         | MCM4          |
| 65 | + | 4.731 | 3.259 | Q15185        | Prostaglandin E synthase 3                                                                    | PTGES3        |
| 66 | + | 8.205 | 3.255 | P15531        | Nucleoside diphosphate kinase A                                                               | NME1          |
| 67 | + | 4.472 | 3.249 | O95433        | Activator of 90 kDa heat shock protein ATPase homolog 1                                       | AHSA1         |
| 68 | + | 3.539 | 3.248 | P32119        | Peroxioredoxin-2                                                                              | PRDX2         |
| 69 | + | 3.226 | 3.189 | P14866        | Heterogeneous nuclear ribonucleoprotein L                                                     | HNRNPL        |
| 70 | + | 5.676 | 3.162 | Q9UHD1        | Cysteine and histidine-rich domain-containing protein 1                                       | CHORDC1       |
| 71 | + | 4.227 | 3.153 | P46940        | Ras GTPase-activating-like protein IQGAP1                                                     | IQGAP1        |
| 72 | + | 7.159 | 3.152 | P61088;Q5JXB2 | Ubiquitin-conjugating enzyme E2 N;Putative ubiquitin-conjugating enzyme E2 N-like             | UBE2N;UBE2NL  |
| 73 | + | 2.807 | 3.142 | Q8NC51        | Plasminogen activator inhibitor 1 RNA-binding protein                                         | SERBP1        |
| 74 | + | 5.278 | 3.114 | P17812        | CTP synthase 1                                                                                | CTPS1         |
| 75 | + | 6.146 | 3.107 | P16152        | Carbonyl reductase [NADPH] 1                                                                  | CBR1          |
| 76 | + | 3.836 | 3.092 | Q15084        | Protein disulfide-isomerase A6                                                                | PDI A6        |
| 77 | + | 4.510 | 3.078 | Q8WUM4        | Programmed cell death 6-interacting protein                                                   | PDCD6IP       |

## Appendix

|     |   |       |       |               |                                                                                                                                                                 |                   |
|-----|---|-------|-------|---------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| 78  | + | 6.650 | 3.067 | O75832        | 26S proteasome non-ATPase regulatory subunit 10                                                                                                                 | PSMD10            |
| 79  | + | 6.554 | 3.040 | Q15785        | Mitochondrial import receptor subunit TOM34                                                                                                                     | TOMM34            |
| 80  | + | 7.649 | 3.039 | P17980        | 26S protease regulatory subunit 6A                                                                                                                              | PSMC3             |
| 81  | + | 7.301 | 3.019 | P13489        | Ribonuclease inhibitor                                                                                                                                          | RNH1              |
| 82  | + | 9.044 | 3.013 | P14618        | Pyruvate kinase PKM                                                                                                                                             | PKM               |
| 83  | + | 6.581 | 2.982 | P31153        | S-adenosylmethionine synthase isoform type-2                                                                                                                    | MAT2A             |
| 84  | + | 6.053 | 2.968 | P41250        | Glycine--tRNA ligase                                                                                                                                            | GARS              |
| 85  | + | 3.083 | 2.965 | P38646        | Stress-70 protein, mitochondrial                                                                                                                                | HSPA9             |
| 86  | + | 3.072 | 2.964 | P47897        | Glutamine--tRNA ligase                                                                                                                                          | QARS              |
| 87  | + | 4.616 | 2.938 | P08243        | Asparagine synthetase [glutamine-hydrolyzing]                                                                                                                   | ASNS              |
| 88  | + | 2.604 | 2.923 | P23396        | 40S ribosomal protein S3                                                                                                                                        | RPS3              |
| 89  | + | 4.823 | 2.907 | P10768        | S-formylglutathione hydrolase                                                                                                                                   | ESD               |
| 90  | + | 4.923 | 2.884 | Q13200        | 26S proteasome non-ATPase regulatory subunit 2                                                                                                                  | PSMD2             |
| 91  | + | 5.270 | 2.852 | O15371        | Eukaryotic translation initiation factor 3 subunit D                                                                                                            | EIF3D             |
| 92  | + | 5.994 | 2.850 | P23921        | Ribonucleoside-diphosphate reductase large subunit                                                                                                              | RRM1              |
| 93  | + | 1.257 | 2.844 | P61604        | 10 kDa heat shock protein, mitochondrial                                                                                                                        | HSPE1             |
| 94  | + | 3.483 | 2.830 | O75369        | Filamin-B                                                                                                                                                       | FLNB              |
| 95  | + | 4.904 | 2.811 | P42704        | Leucine-rich PPR motif-containing protein, mitochondrial                                                                                                        | LRPPRC            |
| 96  | + | 5.097 | 2.807 | O00299        | Chloride intracellular channel protein 1                                                                                                                        | CLIC1             |
| 97  | + | 3.099 | 2.805 | Q04760        | Lactylglutathione lyase                                                                                                                                         | GLO1              |
| 98  | + | 3.845 | 2.798 | P33992        | DNA replication licensing factor MCM5                                                                                                                           | MCM5              |
| 99  | + | 2.756 | 2.789 | P09651;Q32P51 | Heterogeneous nuclear ribonucleoprotein A1;Heterogeneous nuclear ribonucleoprotein A1, N-terminally processed;Heterogeneous nuclear ribonucleoprotein A1-like 2 | HNRNPA1;HNRNPA1L2 |
| 100 | + | 4.889 | 2.782 | Q9Y3F4        | Serine-threonine kinase receptor-associated protein                                                                                                             | STRAP             |
| 101 | + | 5.680 | 2.773 | Q06830        | Peroxioredoxin-1                                                                                                                                                | PRDX1             |
| 102 | + | 4.947 | 2.771 | Q9UHX1        | Poly(U)-binding-splicing factor PUF60                                                                                                                           | PUF60             |
| 103 | + | 5.932 | 2.753 | Q13838        | Spliceosome RNA helicase DDX39B                                                                                                                                 | DDX39B            |
| 104 | + | 3.227 | 2.752 | P55072        | Transitional endoplasmic reticulum ATPase                                                                                                                       | VCP               |
| 105 | + | 4.951 | 2.748 | P35580        | Myosin-10                                                                                                                                                       | MYH10             |

# Identified Proteins

|     |   |        |       |        |                                                                                                                                                                                     |          |
|-----|---|--------|-------|--------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|
| 106 | + | 6.174  | 2.725 | Q9P258 | Protein RCC2                                                                                                                                                                        | RCC2     |
| 107 | + | 2.571  | 2.716 | P29401 | Transketolase                                                                                                                                                                       | TKT      |
| 108 | + | 6.132  | 2.695 | P14868 | Aspartate--tRNA ligase, cytoplasmic                                                                                                                                                 | DARS     |
| 109 | + | 4.644  | 2.694 | O76003 | Glutaredoxin-3                                                                                                                                                                      | GLRX3    |
| 110 | + | 2.613  | 2.680 | Q12905 | Interleukin enhancer-binding factor 2                                                                                                                                               | ILF2     |
| 111 | + | 4.620  | 2.675 | P26196 | Probable ATP-dependent RNA helicase DDX6                                                                                                                                            | DDX6     |
| 112 | + | 2.846  | 2.667 | P07195 | L-lactate dehydrogenase B chain                                                                                                                                                     | LHDB     |
| 113 | + | 3.590  | 2.664 | Q7KZF4 | Staphylococcal nuclease domain-containing protein 1                                                                                                                                 | SND1     |
| 114 | + | 6.778  | 2.658 | P22102 | Trifunctional purine biosynthetic protein adenosine-3;Phosphoribosylamine--glycine ligase;Phosphoribosylformylglycinamide cyclo--ligase;Phosphoribosylglycinamide formyltransferase | GART     |
| 115 | + | 2.460  | 2.641 | P27695 | DNA-(apurinic or apyrimidinic site) lyase;DNA-(apurinic or apyrimidinic site) lyase, mitochondrial                                                                                  | APEX1    |
| 116 | + | 7.326  | 2.638 | O75821 | Eukaryotic translation initiation factor 3 subunit G                                                                                                                                | EIF3G    |
| 117 | + | 5.795  | 2.633 | Q9Y2L1 | Exosome complex exonuclease RRP44                                                                                                                                                   | DIS3     |
| 118 | + | 2.660  | 2.631 | Q00341 | Vigilin                                                                                                                                                                             | HDLBP    |
| 119 | + | 2.081  | 2.623 | P26038 | Moesin                                                                                                                                                                              | MSN      |
| 120 | + | 1.475  | 2.621 | P00558 | Phosphoglycerate kinase 1                                                                                                                                                           | PGK1     |
| 121 | + | 5.807  | 2.613 | P12081 | Histidine--tRNA ligase, cytoplasmic                                                                                                                                                 | HARS     |
| 122 | + | 4.009  | 2.612 | P12004 | Proliferating cell nuclear antigen                                                                                                                                                  | PCNA     |
| 123 | + | 5.253  | 2.601 | Q16543 | Hsp90 co-chaperone Cdc37;Hsp90 co-chaperone Cdc37, N-terminally processed                                                                                                           | CDC37    |
| 124 | + | 3.720  | 2.582 | O43143 | Pre-m RNA-splicing factor ATP-dependent RNA helicase DHX15                                                                                                                          | DHX15    |
| 125 | + | 13.307 | 2.575 | P13639 | Elongation factor 2                                                                                                                                                                 | EEF2     |
| 126 | + | 1.881  | 2.567 | P49915 | GMP synthase [glutamine-hydrolyzing]                                                                                                                                                | GMPS     |
| 127 | + | 4.538  | 2.558 | P12814 | Alpha-actinin-1                                                                                                                                                                     | ACTN1    |
| 128 | + | 5.726  | 2.552 | P36776 | Lon protease homolog, mitochondrial                                                                                                                                                 | LONP1    |
| 129 | + | 4.366  | 2.538 | P26358 | DNA (cytosine-5)-methyltransferase 1                                                                                                                                                | DNMT1    |
| 130 | + | 2.101  | 2.537 | P60174 | Triosephosphate isomerase                                                                                                                                                           | TPI1     |
| 131 | + | 3.099  | 2.535 | P61081 | NEDD8-conjugating enzyme Ubc12                                                                                                                                                      | UBE2M    |
| 132 | + | 6.911  | 2.532 | P08238 | Heat shock protein HSP 90-beta                                                                                                                                                      | HSP90AB1 |
| 133 | + | 2.800  | 2.529 | P23526 | Adenosylhomocysteinase                                                                                                                                                              | AHCY     |

## Appendix

|     |   |       |       |        |                                                                                                     |          |
|-----|---|-------|-------|--------|-----------------------------------------------------------------------------------------------------|----------|
| 134 | + | 2.974 | 2.522 | Q9UMS4 | Pre-mRNA-processing factor 19                                                                       | PRPF19   |
| 135 | + | 3.450 | 2.513 | Q00610 | Clathrin heavy chain 1                                                                              | CLTC     |
| 136 | + | 2.084 | 2.509 | P11940 | Polyadenylate-binding protein 1                                                                     | PABPC1   |
| 137 | + | 4.477 | 2.506 | P55010 | Eukaryotic translation initiation factor 5                                                          | EIF5     |
| 138 | + | 3.265 | 2.493 | Q95373 | Importin-7                                                                                          | IPO7     |
| 139 | + | 2.132 | 2.477 | P05455 | Lupus La protein                                                                                    | SSB      |
| 140 | + | 3.297 | 2.457 | Q13151 | Heterogeneous nuclear ribonucleoprotein A0                                                          | HNRNPA0  |
| 141 | + | 6.981 | 2.448 | P15170 | Eukaryotic peptide chain release factor GTP-binding subunit ERF3A                                   | GSPT1    |
| 142 | + | 1.982 | 2.422 | O75643 | U5 small nuclear ribonucleoprotein 200 kDa helicase                                                 | SNRNP200 |
| 143 | + | 4.468 | 2.413 | P30084 | Enoyl-CoA hydratase, mitochondrial                                                                  | ECHS1    |
| 144 | + | 3.974 | 2.407 | Q96AE4 | Far upstream element-binding protein 1                                                              | FUBP1    |
| 145 | + | 4.741 | 2.406 | P23381 | Tryptophan--tRNA ligase, cytoplasmic;T1-TrpRS;T2-TrpRS                                              | WARS     |
| 146 | + | 2.338 | 2.403 | P43246 | DNA mismatch repair protein Msh2                                                                    | MSH2     |
| 147 | + | 4.335 | 2.396 | P62826 | GTP-binding nuclear protein Ran                                                                     | RAN      |
| 148 | + | 1.748 | 2.391 | P23246 | Splicing factor, proline- and glutamine-rich                                                        | SFPQ     |
| 149 | + | 6.046 | 2.390 | Q9BWD1 | Acetyl-CoA acetyltransferase, cytosolic                                                             | ACAT2    |
| 150 | + | 5.122 | 2.386 | Q9BXJ9 | N-alpha-acetyltransferase 15, NatA auxiliary subunit                                                | NAA15    |
| 151 | + | 5.627 | 2.348 | Q08J23 | tRNA (cytosine(34)-C(5))-methyltransferase                                                          | NSUN2    |
| 152 | + | 2.808 | 2.334 | P62937 | Peptidyl-prolyl cis-trans isomerase A;Peptidyl-prolyl cis-trans isomerase A, N-terminally processed | PIA      |
| 153 | + | 4.169 | 2.327 | P55884 | Eukaryotic translation initiation factor 3 subunit B                                                | EIF3B    |
| 154 | + | 2.585 | 2.311 | Q15233 | Non-POU domain-containing octamer-binding protein                                                   | NONO     |
| 155 | + | 5.754 | 2.306 | O43684 | Mitotic checkpoint protein BUB3                                                                     | BUB3     |
| 156 | + | 4.834 | 2.301 | P60842 | Eukaryotic initiation factor 4A-I                                                                   | EIF4A1   |
| 157 | + | 3.380 | 2.289 | O15067 | Phosphoribosylformylglycinamide synthase                                                            | PFA5     |
| 158 | + | 4.048 | 2.285 | P60228 | Eukaryotic translation initiation factor 3 subunit E                                                | EIF3E    |
| 159 | + | 2.618 | 2.280 | Q9NR30 | Nucleolar RNA helicase 2                                                                            | DDX21    |
| 160 | + | 4.081 | 2.271 | O14929 | Histone acetyltransferase type B catalytic subunit                                                  | HAT1     |
| 161 | + | 5.754 | 2.262 | P50395 | Rab GDP dissociation inhibitor beta                                                                 | GDI2     |
| 162 | + | 3.902 | 2.257 | Q9Y2Z0 | Suppressor of G2 allele of SKP1 homolog                                                             | SUGT1    |

## Identified Proteins

|     |   |       |       |        |                                                                                                                           |         |
|-----|---|-------|-------|--------|---------------------------------------------------------------------------------------------------------------------------|---------|
| 163 | + | 3.074 | 2.250 | P49591 | Serine--tRNA ligase, cytoplasmic                                                                                          | SARS    |
| 164 | + | 2.952 | 2.243 | P07741 | Adenine phosphoribosyltransferase                                                                                         | APRT    |
| 165 | + | 4.819 | 2.241 | P62333 | 26S protease regulatory subunit 10B                                                                                       | PSMC6   |
| 166 | + | 1.925 | 2.220 | P55786 | Puromycin-sensitive aminopeptidase                                                                                        | NPEPPS  |
| 167 | + | 3.979 | 2.208 | Q9Y262 | Eukaryotic translation initiation factor 3 subunit L                                                                      | EIF3L   |
| 168 | + | 4.053 | 2.206 | P20042 | Eukaryotic translation initiation factor 2 subunit 2                                                                      | EIF2S2  |
| 169 | + | 2.876 | 2.177 | P51991 | Heterogeneous nuclear ribonucleoprotein A3                                                                                | HNRNPA3 |
| 170 | + | 1.784 | 2.170 | P31939 | Bifunctional purine biosynthesis protein PURH;Phosphoribosylaminimidazolecarboxamide formyltransferase;IMP cyclohydrolase | ATIC    |
| 171 | + | 4.200 | 2.156 | Q9Y230 | RuvB-like 2                                                                                                               | RUVBL2  |
| 172 | + | 2.604 | 2.153 | P33316 | Deoxyuridine 5-triphosphate nucleotidohydrolase, mitochondrial                                                            | DUT     |
| 173 | + | 3.012 | 2.140 | P24666 | Low molecular weight phosphotyrosine protein phosphatase                                                                  | ACP1    |
| 174 | + | 2.769 | 2.129 | Q9Y6E2 | Basic leucine zipper and W2 domain-containing protein 2                                                                   | BZW2    |
| 175 | + | 2.572 | 2.124 | Q99873 | Protein arginine N-methyltransferase 1                                                                                    | PRMT1   |
| 176 | + | 3.741 | 2.115 | O00148 | ATP-dependent RNA helicase DDX39A                                                                                         | DDX39A  |
| 177 | + | 3.795 | 2.112 | O60841 | Eukaryotic translation initiation factor 5B                                                                               | EIF5B   |
| 178 | + | 5.234 | 2.112 | P62258 | 14-3-3 protein epsilon                                                                                                    | YWHAE   |
| 179 | + | 5.974 | 2.094 | Q5VYK3 | Proteasome-associated protein ECM29 homolog                                                                               | ECM29   |
| 180 | + | 1.378 | 2.090 | P19338 | Nucleolin                                                                                                                 | NCL     |
| 181 | + | 4.174 | 2.087 | P48643 | T-complex protein 1 subunit epsilon                                                                                       | CCT5    |
| 182 | + | 1.180 | 2.084 | Q15056 | Eukaryotic translation initiation factor 4H                                                                               | EIF4H   |
| 183 | + | 6.365 | 2.053 | Q9NZI8 | Insulin-like growth factor 2 mRNA-binding protein 1                                                                       | IGF2BP1 |
| 184 | + | 5.698 | 2.042 | P49411 | Elongation factor Tu, mitochondrial                                                                                       | TUFM    |
| 185 | + | 5.493 | 2.039 | O43242 | 26S proteasome non-ATPase regulatory subunit 3                                                                            | PSMD3   |
| 186 | + | 4.357 | 2.035 | Q7L2H7 | Eukaryotic translation initiation factor 3 subunit M                                                                      | EIF3M   |
| 187 | + | 6.608 | 2.019 | P62191 | 26S protease regulatory subunit 4                                                                                         | PSMC1   |
| 188 | + | 4.327 | 2.013 | O60506 | Heterogeneous nuclear ribonucleoprotein Q                                                                                 | SYNCRIP |
| 189 | + | 5.129 | 2.013 | P48739 | Phosphatidylinositol transfer protein beta isoform                                                                        | PITPNB  |
| 190 | + | 5.007 | 1.986 | P07737 | Profilin-1                                                                                                                | PFN1    |

## Appendix

|     |   |       |       |               |                                                                                                                           |         |
|-----|---|-------|-------|---------------|---------------------------------------------------------------------------------------------------------------------------|---------|
| 191 | + | 4.033 | 1.965 | P23528        | Cofilin-1                                                                                                                 | CFL1    |
| 192 | + | 2.371 | 1.963 | Q8TEX9        | Importin-4                                                                                                                | IPO4    |
| 193 | + | 5.332 | 1.958 | P0DN79;P35520 | Cystathionine beta-synthase                                                                                               | CBS     |
| 194 | + | 4.742 | 1.934 | P33176        | Kinesin-1 heavy chain                                                                                                     | KIF5B   |
| 195 | + | 1.755 | 1.929 | P06737        | Glycogen phosphorylase, liver form                                                                                        | PYGL    |
| 196 | + | 2.051 | 1.925 | P61247        | 40S ribosomal protein S3a                                                                                                 | RPS3A   |
| 197 | + | 5.072 | 1.924 | Q14152        | Eukaryotic translation initiation factor 3 subunit A                                                                      | EIF3A   |
| 198 | + | 1.200 | 1.917 | P29692        | Elongation factor 1-delta                                                                                                 | EEF1D   |
| 199 | + | 5.702 | 1.912 | Q13642        | Four and a half LIM domains protein 1                                                                                     | FHL1    |
| 200 | + | 4.691 | 1.909 | Q9NP79        | Vacuolar protein sorting-associated protein VTA1 homolog                                                                  | VTA1    |
| 201 | + | 2.331 | 1.901 | O75534        | Cold shock domain-containing protein E1                                                                                   | CSDE1   |
| 202 | + | 5.881 | 1.890 | P63261        | Actin, cytoplasmic 2;Actin, cytoplasmic 2, N-terminally processed                                                         | ACTG1   |
| 203 | + | 3.581 | 1.877 | P35998        | 26S protease regulatory subunit 7                                                                                         | PSMC2   |
| 204 | + | 1.285 | 1.870 | P04181        | Ornithine aminotransferase, mitochondrial;Ornithine aminotransferase, hepatic form;Ornithine aminotransferase, renal form | OAT     |
| 205 | + | 2.713 | 1.861 | P11413        | Glucose-6-phosphate 1-dehydrogenase                                                                                       | G6PD    |
| 206 | + | 3.188 | 1.849 | Q86VP6        | Cullin-associated NEDD8-dissociated protein 1                                                                             | CAND1   |
| 207 | + | 3.284 | 1.845 | P08559        | Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial                                            | PDHA1   |
| 208 | + | 4.426 | 1.826 | P43897        | Elongation factor Ts, mitochondrial                                                                                       | TSFM    |
| 209 | + | 1.772 | 1.822 | O75937        | DnaJ homolog subfamily C member 8                                                                                         | DNAJC8  |
| 210 | + | 5.499 | 1.821 | P61978        | Heterogeneous nuclear ribonucleoprotein K                                                                                 | HNRNPK  |
| 211 | + | 1.940 | 1.815 | P31689        | DnaJ homolog subfamily A member 1                                                                                         | DNAJA1  |
| 212 | + | 3.467 | 1.811 | Q8N1G4        | Leucine-rich repeat-containing protein 47                                                                                 | LRRC47  |
| 213 | + | 4.303 | 1.805 | P60981        | Destrin                                                                                                                   | DSTN    |
| 214 | + | 4.155 | 1.799 | P50990        | T-complex protein 1 subunit theta                                                                                         | CCT8    |
| 215 | + | 2.740 | 1.794 | Q15393        | Splicing factor 3B subunit 3                                                                                              | SF3B3   |
| 216 | + | 2.929 | 1.794 | P31942        | Heterogeneous nuclear ribonucleoprotein H3                                                                                | HNRNPH3 |
| 217 | + | 2.956 | 1.778 | P11172        | Uridine 5-monophosphate synthase;Orotate phosphoribosyltransferase;Orotidine 5-phosphate decarboxylase                    | UMPS    |

## Identified Proteins

|     |   |       |       |        |                                                                                                            |          |
|-----|---|-------|-------|--------|------------------------------------------------------------------------------------------------------------|----------|
| 218 | + | 2.305 | 1.776 | O43776 | Asparagine--tRNA ligase, cytoplasmic                                                                       | NARS     |
| 219 | + | 4.260 | 1.771 | O43175 | D-3-phosphoglycerate dehydrogenase                                                                         | PHGDH    |
| 220 | + | 1.763 | 1.770 | Q13283 | Ras GTPase-activating protein-binding protein 1                                                            | G3BP1    |
| 221 | + | 3.162 | 1.766 | P63104 | 14-3-3 protein zeta/delta                                                                                  | YWHAZ    |
| 222 | + | 1.393 | 1.757 | P00492 | Hypoxanthine-guanine phosphoribosyltransferase                                                             | HPRT1    |
| 223 | + | 4.064 | 1.743 | O00154 | Cytosolic acyl coenzyme A thioester hydrolase                                                              | ACOT7    |
| 224 | + | 4.414 | 1.743 | Q15691 | Microtubule-associated protein RP/EB family member 1                                                       | MAPRE1   |
| 225 | + | 4.778 | 1.735 | Q00839 | Heterogeneous nuclear ribonucleoprotein U                                                                  | HNRNP-U  |
| 226 | + | 1.460 | 1.725 | P61158 | Actin-related protein 3                                                                                    | ACTR3    |
| 227 | + | 1.602 | 1.723 | O75874 | Isocitrate dehydrogenase [NADP] cytoplasmic                                                                | IDH1     |
| 228 | + | 1.389 | 1.707 | P62633 | Cellular nucleic acid-binding protein                                                                      | CNBP     |
| 229 | + | 2.721 | 1.697 | Q9NPD8 | Ubiquitin-conjugating enzyme E2 T                                                                          | UBE2T    |
| 230 | + | 2.557 | 1.695 | P55209 | Nucleosome assembly protein 1-like 1                                                                       | NAP1L1   |
| 231 | + | 4.088 | 1.693 | P49419 | Alpha-amino adipic semialdehyde dehydrogenase                                                              | ALDH7A1  |
| 232 | + | 1.414 | 1.692 | P50454 | Serpin H1                                                                                                  | SERPINH1 |
| 233 | + | 3.119 | 1.678 | Q9Y4L1 | Hypoxia up-regulated protein 1                                                                             | HYOU1    |
| 234 | + | 1.785 | 1.678 | P62913 | 60S ribosomal protein L11                                                                                  | RPL11    |
| 235 | + | 6.955 | 1.660 | P49327 | Fatty acid synthase:[Acyl-carrier-protein] S-acetyltransferase:[Acyl-carrier-protein] S-malonyltransferase | FASN     |
| 236 | + | 1.869 | 1.655 | Q15459 | Splicing factor 3A subunit 1                                                                               | SF3A1    |
| 237 | + | 5.164 | 1.654 | Q9BXP5 | Serrate RNA effector molecule homolog                                                                      | SRRT     |
| 238 | + | 3.376 | 1.653 | Q9C0C9 | E2/E3 hybrid ubiquitin-protein ligase UBE2O                                                                | UBE2O    |
| 239 | + | 6.749 | 1.645 | Q9Y5K5 | Ubiquitin carboxyl-terminal hydrolase isozyme L5                                                           | UCHL5    |
| 240 | + | 2.807 | 1.623 | P45974 | Ubiquitin carboxyl-terminal hydrolase 5                                                                    | USP5     |
| 241 | + | 4.244 | 1.600 | Q15717 | ELAV-like protein 1                                                                                        | ELAVL1   |
| 242 | + | 2.772 | 1.599 | Q96P70 | Importin-9                                                                                                 | IPO9     |
| 243 | + | 5.172 | 1.598 | P46777 | 60S ribosomal protein L5                                                                                   | RPL5     |
| 244 | + | 2.666 | 1.582 | Q9Y450 | HBS1-like protein                                                                                          | HBS1L    |
| 245 | + | 3.743 | 1.581 | Q08211 | ATP-dependent RNA helicase A                                                                               | DHX9     |

|     |   |       |       |        |                                                                                                                                           |          |
|-----|---|-------|-------|--------|-------------------------------------------------------------------------------------------------------------------------------------------|----------|
| 246 | + | 2.479 | 1.574 | Q9BPX3 | Condensin complex subunit 3                                                                                                               | NCAPG    |
| 247 | + | 2.492 | 1.567 | P63244 | Guanine nucleotide-binding protein subunit beta-2-like 1;Guanine nucleotide-binding protein subunit beta-2-like 1, N-terminally processed | GNB2L1   |
| 248 | + | 2.067 | 1.560 | Q14204 | Cytoplasmic dynein 1 heavy chain 1                                                                                                        | DYNC1H1  |
| 249 | + | 2.947 | 1.559 | Q9NRN7 | L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase                                                                 | AASDHPPT |
| 250 | + | 2.200 | 1.540 | Q9NUU7 | ATP-dependent RNA helicase DDX19A                                                                                                         | DDX19A   |
| 251 | + | 3.255 | 1.536 | O43681 | ATPase ASNA1                                                                                                                              | ASNA1    |
| 252 | + | 3.215 | 1.536 | Q9Y295 | Developmentally-regulated GTP-binding protein 1                                                                                           | DRG1     |
| 253 | + | 2.236 | 1.524 | Q14683 | Structural maintenance of chromosomes protein 1A                                                                                          | SMC1A    |
| 254 | + | 5.132 | 1.523 | Q13263 | Transcription intermediary factor 1-beta                                                                                                  | TRIM28   |
| 255 | + | 2.976 | 1.486 | O95801 | Tetratricopeptide repeat protein 4                                                                                                        | TTC4     |
| 256 | + | 4.327 | 1.452 | O75153 | Clustered mitochondria protein homolog                                                                                                    | CLUH     |
| 257 | + | 4.862 | 1.445 | Q6P148 | Aspartate--tRNA ligase, mitochondrial                                                                                                     | DARS2    |
| 258 | + | 4.774 | 1.441 | Q15008 | 26S proteasome non-ATPase regulatory subunit 6                                                                                            | PSMD6    |
| 259 | + | 4.074 | 1.399 | Q7Z6Z7 | E3 ubiquitin-protein ligase HUWE1                                                                                                         | HUWE1    |

2.2 Proteins Significantly Enriched in the Competition Experiment in Comparison to the Beads Control

Table 8: Proteins significantly enriched in the competition experiment in comparison to the beads control.

|   | Significant | -log p | Difference | Majority protein IDs | Protein names                                                                               | Gene names |
|---|-------------|--------|------------|----------------------|---------------------------------------------------------------------------------------------|------------|
| 1 | +           | 12.107 | 5.164      | P04406               | Glyceraldehyde-3-phosphate dehydrogenase                                                    | GAPDH      |
| 2 | +           | 5.706  | 5.045      | Q9Y490               | Talin-1                                                                                     | TLN1       |
| 3 | +           | 3.384  | 5.011      | P12277               | Creatine kinase B-type                                                                      | CKB        |
| 4 | +           | 4.738  | 4.941      | P30086               | Phosphatidylethanolamine-binding protein 1;Hippocampal cholinergic neurostimulating peptide | PEBP1      |
| 5 | +           | 5.128  | 4.663      | Q9HB71               | Calcyclin-binding protein                                                                   | CACYBP     |
| 6 | +           | 3.608  | 4.636      | P14625               | Endoplasmic                                                                                 | HSP90B1    |
| 7 | +           | 3.706  | 4.538      | P34932               | Heat shock 70 kDa protein 4                                                                 | HSPA4      |
| 8 | +           | 5.043  | 4.366      | Q92598               | Heat shock protein 105 kDa                                                                  | HSPH1      |

## Identified Proteins

|    |   |       |       |        |                                                                                                             |           |
|----|---|-------|-------|--------|-------------------------------------------------------------------------------------------------------------|-----------|
| 9  | + | 6.544 | 4.277 | P61221 | ATP-binding cassette sub-family E member 1                                                                  | ABCE1     |
| 10 | + | 3.753 | 4.249 | P08865 | 40S ribosomal protein SA                                                                                    | RPSA      |
| 11 | + | 4.038 | 4.226 | Q14103 | Heterogeneous nuclear ribonucleoprotein D0                                                                  | HNRNPD    |
| 12 | + | 6.600 | 4.216 | P21333 | Filamin-A                                                                                                   | FLNA      |
| 13 | + | 5.947 | 4.200 | P29401 | Transketolase                                                                                               | TKT       |
| 14 | + | 4.668 | 4.149 | P13797 | Plastin-3                                                                                                   | PLS3      |
| 15 | + | 8.229 | 4.116 | P27348 | 14-3-3 protein theta                                                                                        | YWHAQ     |
| 16 | + | 6.506 | 4.102 | Q14166 | Tubulin--tyrosine ligase-like protein 12                                                                    | TTL12     |
| 17 | + | 7.234 | 4.096 | Q9P2J5 | Leucine--tRNA ligase, cytoplasmic                                                                           | LARS      |
| 18 | + | 3.136 | 4.039 | P31948 | Stress-induced-phosphoprotein 1                                                                             | STIP1     |
| 19 | + | 5.438 | 4.020 | Q15181 | Inorganic pyrophosphatase                                                                                   | PPA1      |
| 20 | + | 7.821 | 4.003 | P33993 | DNA replication licensing factor MCM7                                                                       | MCM7      |
| 21 | + | 3.341 | 3.995 | P37802 | Transgelin-2                                                                                                | TAGLN2    |
| 22 | + | 5.337 | 3.978 | P35579 | Myosin-9                                                                                                    | MYH9      |
| 23 | + | 3.200 | 3.978 | Q02790 | Peptidyl-prolyl cis-trans isomerase FKBP4;Peptidyl-prolyl cis-trans isomerase FKBP4, N-terminally processed | FKBP4     |
| 24 | + | 4.866 | 3.953 | O95433 | Activator of 90 kDa heat shock protein ATPase homolog 1                                                     | AHSA1     |
| 25 | + | 3.215 | 3.952 | Q13162 | Peroxiredoxin-4                                                                                             | PRDX4     |
| 26 | + | 4.244 | 3.930 | P06733 | Alpha-enolase                                                                                               | ENO1      |
| 27 | + | 4.226 | 3.916 | P07900 | Heat shock protein HSP 90-alpha                                                                             | HSP90AA1  |
| 28 | + | 5.182 | 3.913 | Q9UHD1 | Cysteine and histidine-rich domain-containing protein 1                                                     | CHORDC1   |
| 29 | + | 3.285 | 3.877 | P22626 | Heterogeneous nuclear ribonucleoproteins A2/B1                                                              | HNRNPA2B1 |
| 30 | + | 6.409 | 3.794 | P26640 | Valine--tRNA ligase                                                                                         | VARS      |
| 31 | + | 6.020 | 3.793 | Q14566 | DNA replication licensing factor MCM6                                                                       | MCM6      |
| 32 | + | 3.699 | 3.791 | P22314 | Ubiquitin-like modifier-activating enzyme 1                                                                 | UBA1      |
| 33 | + | 2.762 | 3.790 | P07237 | Protein disulfide-isomerase                                                                                 | P4HB      |
| 34 | + | 3.066 | 3.786 | P55786 | Puromycin-sensitive aminopeptidase                                                                          | NPEPPS    |
| 35 | + | 5.118 | 3.724 | P54136 | Arginine--tRNA ligase, cytoplasmic                                                                          | RARS      |
| 36 | + | 5.148 | 3.705 | P23284 | Peptidyl-prolyl cis-trans isomerase B                                                                       | PIIB      |

## Appendix

|    |   |       |       |        |                                                                                                                                |        |
|----|---|-------|-------|--------|--------------------------------------------------------------------------------------------------------------------------------|--------|
| 37 | + | 2.788 | 3.699 | P32119 | Peroxioredoxin-2                                                                                                               | PRDX2  |
| 38 | + | 4.283 | 3.690 | P53396 | ATP-citrate synthase                                                                                                           | ACLY   |
| 39 | + | 4.224 | 3.686 | P00338 | L-lactate dehydrogenase A chain                                                                                                | LDHA   |
| 40 | + | 4.626 | 3.665 | P33991 | DNA replication licensing factor MCM4                                                                                          | MCM4   |
| 41 | + | 5.054 | 3.651 | Q16543 | Hsp90 co-chaperone Cdc37;Hsp90 co-chaperone Cdc37, N-terminally processed                                                      | CDC37  |
| 42 | + | 4.244 | 3.631 | Q15084 | Protein disulfide-isomerase A6                                                                                                 | PDIA6  |
| 43 | + | 7.150 | 3.599 | P56192 | Methionine--tRNA ligase, cytoplasmic                                                                                           | MARS   |
| 44 | + | 5.842 | 3.558 | P26641 | Elongation factor 1--gamma                                                                                                     | EEF1G  |
| 45 | + | 3.139 | 3.550 | P40926 | Malate dehydrogenase, mitochondrial                                                                                            | MDH2   |
| 46 | + | 6.706 | 3.507 | P25205 | DNA replication licensing factor MCM3                                                                                          | MCM3   |
| 47 | + | 4.481 | 3.506 | P54577 | Tyrosine--tRNA ligase, cytoplasmic;Tyrosine--tRNA ligase, cytoplasmic, N-terminally processed                                  | YARS   |
| 48 | + | 5.107 | 3.426 | Q9UHX1 | Poly(U)-binding-splicing factor PUF60                                                                                          | PUF60  |
| 49 | + | 6.216 | 3.413 | Q9UQ80 | Proliferation-associated protein 2G4                                                                                           | PA2G4  |
| 50 | + | 1.976 | 3.409 | P61604 | 10 kDa heat shock protein, mitochondrial                                                                                       | HSPE1  |
| 51 | + | 3.202 | 3.375 | P04075 | Fructose-bisphosphate aldolase A                                                                                               | ALDOA  |
| 52 | + | 5.253 | 3.364 | P49736 | DNA replication licensing factor MCM2                                                                                          | MCM2   |
| 53 | + | 3.042 | 3.362 | P30101 | Protein disulfide-isomerase A3                                                                                                 | PDIA3  |
| 54 | + | 3.483 | 3.307 | P30048 | Thioredoxin-dependent peroxide reductase, mitochondrial                                                                        | PRDX3  |
| 55 | + | 5.825 | 3.304 | Q15185 | Prostaglandin H synthase 3                                                                                                     | PTGES3 |
| 56 | + | 6.017 | 3.303 | O43390 | Heterogeneous nuclear ribonucleoprotein R                                                                                      | HNRNPR |
| 57 | + | 4.515 | 3.295 | Q04760 | Lactoylglutathione lyase                                                                                                       | GLO1   |
| 58 | + | 4.338 | 3.278 | P22234 | Multifunctional protein ADE2;Phosphoribosylaminoimidazole-succinocarboxamide synthase;Phosphoribosylaminoimidazole carboxylase | PAICS  |
| 59 | + | 4.990 | 3.226 | O75832 | 26S proteasome non-ATPase regulatory subunit 10                                                                                | PSMD10 |
| 60 | + | 3.247 | 3.203 | Q8NC51 | Plasminogen activator inhibitor 1 RNA-binding protein                                                                          | SERBP1 |
| 61 | + | 4.199 | 3.199 | P07814 | Bifunctional glutamate/proline--tRNA ligase;Glutamate--tRNA ligase;Proline--tRNA ligase                                        | EPRS   |
| 62 | + | 1.729 | 3.187 | P12268 | Inosine-5-monophosphate dehydrogenase 2                                                                                        | IMPDH2 |
| 63 | + | 4.148 | 3.126 | P33992 | DNA replication licensing factor MCM5                                                                                          | MCM5   |
| 64 | + | 5.563 | 3.122 | P12814 | Alpha-actinin-1                                                                                                                | ACTN1  |

## Identified Proteins

|    |   |        |       |               |                                                                                                                                                                 |                   |
|----|---|--------|-------|---------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| 65 | + | 4.376  | 3.115 | P10768        | S-formylglutathione hydrolase                                                                                                                                   | ESD               |
| 66 | + | 7.611  | 3.113 | P62136        | Serine/threonine-protein phosphatase PP1-alpha catalytic subunit                                                                                                | PPP1CA            |
| 67 | + | 10.250 | 3.096 | P14618        | Pyruvate kinase PKM                                                                                                                                             | PKM               |
| 68 | + | 3.723  | 3.090 | P07195        | L-lactate dehydrogenase B chain                                                                                                                                 | LDHB              |
| 69 | + | 4.031  | 3.064 | P08243        | Asparagine synthetase [glutamine-hydrolyzing]                                                                                                                   | ASNS              |
| 70 | + | 4.162  | 3.035 | P17812        | CTP synthase 1                                                                                                                                                  | CTPS1             |
| 71 | + | 6.568  | 3.009 | P62937        | Peptidyl-prolyl cis-trans isomerase A;Peptidyl-prolyl cis-trans isomerase A, N-terminally processed                                                             | PIPA              |
| 72 | + | 5.490  | 3.008 | P46940        | Ras GTPase-activating-like protein IQGAP1                                                                                                                       | IQGAP1            |
| 73 | + | 6.823  | 2.978 | P41252        | Isoleucine--tRNA ligase, cytoplasmic                                                                                                                            | IARS              |
| 74 | + | 5.469  | 2.961 | Q9Y3F4        | Serine-threonine kinase receptor-associated protein                                                                                                             | STRAP             |
| 75 | + | 2.785  | 2.953 | P19338        | Nucleolin                                                                                                                                                       | NCL               |
| 76 | + | 2.945  | 2.949 | P23396        | 40S ribosomal protein S3                                                                                                                                        | RPS3              |
| 77 | + | 5.090  | 2.945 | Q15785        | Mitochondrial import receptor subunit TOM34                                                                                                                     | TOMM34            |
| 78 | + | 3.955  | 2.925 | P06493        | Cyclin-dependent kinase 1                                                                                                                                       | CDK1              |
| 79 | + | 5.146  | 2.924 | P62495        | Eukaryotic peptide chain release factor subunit 1                                                                                                               | ETF1              |
| 80 | + | 4.876  | 2.916 | Q9Y265        | RuvB-like 1                                                                                                                                                     | RUVBL1            |
| 81 | + | 2.004  | 2.904 | Q14697        | Neutral alpha-glucosidase AB                                                                                                                                    | GANAB             |
| 82 | + | 4.782  | 2.887 | P27695        | DNA-(apurinic or apyrimidinic site) lyase;DNA-(apurinic or apyrimidinic site) lyase, mitochondrial                                                              | APEX1             |
| 83 | + | 3.946  | 2.881 | O75369        | Filamin-B                                                                                                                                                       | FLNB              |
| 84 | + | 4.181  | 2.864 | O75937        | DnaJ homolog subfamily C member 8                                                                                                                               | DNAJC8            |
| 85 | + | 4.268  | 2.835 | O15371        | Eukaryotic translation initiation factor 3 subunit D                                                                                                            | EIF3D             |
| 86 | + | 6.308  | 2.834 | P16152        | Carbonyl reductase [NADPH] 1                                                                                                                                    | CBR1              |
| 87 | + | 6.419  | 2.828 | P35580        | Myosin-10                                                                                                                                                       | MYH10             |
| 88 | + | 7.922  | 2.815 | O76003        | Glutaredoxin-3                                                                                                                                                  | GLRX3             |
| 89 | + | 5.402  | 2.801 | P48739        | Phosphatidylinositol transfer protein beta isoform                                                                                                              | PITPNB            |
| 90 | + | 5.817  | 2.789 | P50454        | Serpin H1                                                                                                                                                       | SERPINH1          |
| 91 | + | 3.902  | 2.780 | P62837;P61077 | Ubiquitin-conjugating enzyme E2 D2;Ubiquitin-conjugating enzyme E2 D3                                                                                           | UBE2D2;UBE2D3     |
| 92 | + | 1.800  | 2.778 | P09651;Q32P51 | Heterogeneous nuclear ribonucleoprotein A1;Heterogeneous nuclear ribonucleoprotein A1, N-terminally processed;Heterogeneous nuclear ribonucleoprotein A1-like 2 | HNRNPA1;HNRNPA1L2 |

## Appendix

|     |   |  |        |       |        |                                                                                                 |  |          |
|-----|---|--|--------|-------|--------|-------------------------------------------------------------------------------------------------|--|----------|
| 93  | + |  | 7.527  | 2.777 | Q9P258 | Protein RCC2                                                                                    |  | RCC2     |
| 94  | + |  | 4.119  | 2.763 | P23381 | Tryptophan--tRNA ligase, cytoplasmic;T1-TrpRS;T2-TrpRS                                          |  | WARS     |
| 95  | + |  | 2.388  | 2.757 | P00505 | Aspartate aminotransferase, mitochondrial                                                       |  | GOT2     |
| 96  | + |  | 4.514  | 2.748 | P49588 | Alanine--tRNA ligase, cytoplasmic                                                               |  | AARS     |
| 97  | + |  | 4.703  | 2.732 | P42704 | Leucine-rich PPR motif-containing protein, mitochondrial                                        |  | LRPPRC   |
| 98  | + |  | 4.907  | 2.731 | P38919 | Eukaryotic initiation factor 4A-III;Eukaryotic initiation factor 4A-III, N-terminally processed |  | EIF4A3   |
| 99  | + |  | 3.090  | 2.714 | O43776 | Asparagine--tRNA ligase, cytoplasmic                                                            |  | NARS     |
| 100 | + |  | 7.714  | 2.688 | P08238 | Heat shock protein HSP 90-beta                                                                  |  | HSP90AB1 |
| 101 | + |  | 5.957  | 2.684 | O75821 | Eukaryotic translation initiation factor 3 subunit G                                            |  | EIF3G    |
| 102 | + |  | 3.424  | 2.680 | Q8WUJ4 | Programmed cell death 6-interacting protein                                                     |  | PDCD6IP  |
| 103 | + |  | 7.557  | 2.679 | P23921 | Ribonucleoside-diphosphate reductase large subunit                                              |  | RRM1     |
| 104 | + |  | 2.148  | 2.649 | P29692 | Elongation factor 1-delta                                                                       |  | EEF1D    |
| 105 | + |  | 6.858  | 2.628 | Q96AE4 | Far upstream element-binding protein 1                                                          |  | FUBP1    |
| 106 | + |  | 7.047  | 2.627 | P18206 | Vinculin                                                                                        |  | VCL      |
| 107 | + |  | 3.259  | 2.620 | Q99873 | Protein arginine N-methyltransferase 1                                                          |  | PRMT1    |
| 108 | + |  | 1.911  | 2.609 | P49915 | GMP synthase [glutamine-hydrolyzing]                                                            |  | GMPS     |
| 109 | + |  | 2.432  | 2.602 | P26038 | Moesin                                                                                          |  | MSN      |
| 110 | + |  | 5.515  | 2.602 | P18754 | Regulator of chromosome condensation                                                            |  | RCC1     |
| 111 | + |  | 4.516  | 2.597 | P13489 | Ribonuclease inhibitor                                                                          |  | RNH1     |
| 112 | + |  | 3.220  | 2.595 | Q15056 | Eukaryotic translation initiation factor 4H                                                     |  | EIF4H    |
| 113 | + |  | 3.751  | 2.594 | P45974 | Ubiquitin carboxyl-terminal hydrolase 5                                                         |  | USP5     |
| 114 | + |  | 1.681  | 2.590 | P13667 | Protein disulfide-isomerase A4                                                                  |  | PDI4A    |
| 115 | + |  | 4.339  | 2.578 | P33176 | Kinesin-1 heavy chain                                                                           |  | KIF5B    |
| 116 | + |  | 5.005  | 2.576 | Q7L1Q6 | Basic leucine zipper and W2 domain-containing protein 1                                         |  | BZW1     |
| 117 | + |  | 5.062  | 2.561 | P26196 | Probable ATP-dependent RNA helicase DDX6                                                        |  | DDX6     |
| 118 | + |  | 12.089 | 2.539 | P13639 | Elongation factor 2                                                                             |  | EEF2     |
| 119 | + |  | 3.395  | 2.537 | P05455 | Lupus La protein                                                                                |  | SSB      |
| 120 | + |  | 2.763  | 2.521 | Q7KZF4 | Staphylococcal nuclease domain-containing protein 1                                             |  | SND1     |
| 121 | + |  | 2.520  | 2.511 | P11940 | Polyadenylate-binding protein 1                                                                 |  | PABPC1   |

## Identified Proteins

|     |   |       |       |               |                                                                                                                                                                                     |               |
|-----|---|-------|-------|---------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| 122 | + | 4.488 | 2.486 | Q06830        | Peroxioredoxin-1                                                                                                                                                                    | PRDX1         |
| 123 | + | 4.826 | 2.472 | P41250        | Glycine--tRNA ligase                                                                                                                                                                | GARS          |
| 124 | + | 6.939 | 2.459 | P22102        | Trifunctional purine biosynthetic protein adenosine-3;Phosphoribosylamine--glycine ligase;Phosphoribosylformylglycinamide cyclo- ligase;Phosphoribosylglycinamide formyltransferase | GART          |
| 125 | + | 6.921 | 2.454 | P24666        | Low molecular weight phosphotyrosine protein phosphatase                                                                                                                            | ACP1          |
| 126 | + | 2.697 | 2.454 | Q12906        | Interleukin enhancer-binding factor 3                                                                                                                                               | ILF3          |
| 127 | + | 4.670 | 2.451 | P31939        | Bifunctional purine biosynthesis protein PURH;Phosphoribosylaminimidazolecarboxamide formyltransferase;IMP cyclohydrolase                                                           | ATIC          |
| 128 | + | 4.496 | 2.451 | P61088;Q5JXB2 | Ubiquitin-conjugating enzyme E2 N;Putative ubiquitin-conjugating enzyme E2 N-like                                                                                                   | UBE2N;UBE2NL  |
| 129 | + | 2.538 | 2.447 | P31689        | DnaI homolog subfamily A member 1                                                                                                                                                   | DNAJA1        |
| 130 | + | 6.263 | 2.447 | P60891        | Ribose-phosphate pyrophosphokinase 1                                                                                                                                                | PRPS1         |
| 131 | + | 5.247 | 2.446 | P14868        | Aspartate--tRNA ligase, cytoplasmic                                                                                                                                                 | DARS          |
| 132 | + | 4.852 | 2.445 | Q06203        | Amidophosphoribosyltransferase                                                                                                                                                      | PPAT          |
| 133 | + | 5.435 | 2.443 | Q9NP79        | Vacuolar protein sorting-associated protein VTA1 homolog                                                                                                                            | VTA1          |
| 134 | + | 3.287 | 2.428 | P47897        | Glutamine--tRNA ligase                                                                                                                                                              | QARS          |
| 135 | + | 5.445 | 2.406 | P26358        | DNA (cytosine-5)-methyltransferase 1                                                                                                                                                | DNMT1         |
| 136 | + | 4.196 | 2.393 | P17980        | 26S protease regulatory subunit 6A                                                                                                                                                  | PSMC3         |
| 137 | + | 7.493 | 2.390 | P63244        | Guanine nucleotide-binding protein subunit beta-2-like 1;Guanine nucleotide-binding protein subunit beta-2-like 1, N-terminally processed                                           | GNB2L1        |
| 138 | + | 3.722 | 2.385 | Q43143        | Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15                                                                                                                           | DHX15         |
| 139 | + | 4.102 | 2.382 | O60506        | Heterogeneous nuclear ribonucleoprotein Q                                                                                                                                           | SYNCRIP       |
| 140 | + | 2.357 | 2.352 | P00558        | Phosphoglycerate kinase 1                                                                                                                                                           | PGK1          |
| 141 | + | 8.804 | 2.348 | P36776        | Lon protease homolog, mitochondrial                                                                                                                                                 | LONP1         |
| 142 | + | 4.727 | 2.313 | P42771;P42772 | Cyclin-dependent kinase inhibitor 2A;Cyclin-dependent kinase 4 inhibitor B                                                                                                          | CDKN2A;CDKN2B |
| 143 | + | 3.246 | 2.294 | P35637;Q92804 | RNA-binding protein FUS;TATA-binding protein-associated factor 2N                                                                                                                   | FUS;TAF15     |
| 144 | + | 3.406 | 2.287 | P31942        | Heterogeneous nuclear ribonucleoprotein H3                                                                                                                                          | HNRNPH3       |
| 145 | + | 1.211 | 2.282 | Q58FF8        | Putative heat shock protein HSP 90-beta 2                                                                                                                                           | HSP90AB2P     |
| 146 | + | 4.831 | 2.280 | P55884        | Eukaryotic translation initiation factor 3 subunit B                                                                                                                                | EIF3B         |
| 147 | + | 2.888 | 2.279 | P06737        | Glycogen phosphorylase, liver form                                                                                                                                                  | PYGL          |

## Appendix

|     |   |       |       |        |                                                                                                        |         |
|-----|---|-------|-------|--------|--------------------------------------------------------------------------------------------------------|---------|
| 148 | + | 4.112 | 2.273 | P11172 | Uridine 5-monophosphate synthase;Orotate phosphoribosyltransferase;Orotidine 5-phosphate decarboxylase | UMPS    |
| 149 | + | 5.976 | 2.272 | Q13200 | 26S proteasome non-ATPase regulatory subunit 2                                                         | PSMD2   |
| 150 | + | 3.917 | 2.271 | O95373 | Importin-7                                                                                             | IPO7    |
| 151 | + | 1.382 | 2.270 | P61158 | Actin-related protein 3                                                                                | ACTR3   |
| 152 | + | 2.503 | 2.264 | P14866 | Heterogeneous nuclear ribonucleoprotein L                                                              | HNRNPL  |
| 153 | + | 2.852 | 2.254 | P11021 | 78 kDa glucose-regulated protein                                                                       | HSPA5   |
| 154 | + | 6.018 | 2.253 | P49411 | Elongation factor Tu, mitochondrial                                                                    | TUFM    |
| 155 | + | 4.540 | 2.247 | O43242 | 26S proteasome non-ATPase regulatory subunit 3                                                         | PSMD3   |
| 156 | + | 2.131 | 2.244 | P38646 | Stress-70 protein, mitochondrial                                                                       | HSPA9   |
| 157 | + | 2.807 | 2.239 | Q9UMS4 | Pre-mRNA-processing factor 19                                                                          | PRPF19  |
| 158 | + | 3.952 | 2.239 | P31153 | S-adenosylmethionine synthase isoform type-2                                                           | MAT2A   |
| 159 | + | 1.620 | 2.236 | P61081 | NEDD8-conjugating enzyme Ubc12                                                                         | UBE2M   |
| 160 | + | 1.380 | 2.230 | P14314 | Glucosidase 2 subunit beta                                                                             | PRKCSH  |
| 161 | + | 2.715 | 2.229 | O15067 | Phosphoribosylformylglycinamide synthase                                                               | PFAS    |
| 162 | + | 5.383 | 2.201 | P00918 | Carbonic anhydrase 2                                                                                   | CA2     |
| 163 | + | 3.907 | 2.199 | Q9C0C9 | E2/E3 hybrid ubiquitin-protein ligase UBE2O                                                            | UBE2O   |
| 164 | + | 3.339 | 2.197 | Q08123 | tRNA (cytosine(34)-C(5))-methyltransferase                                                             | NSUN2   |
| 165 | + | 1.768 | 2.195 | P11142 | Heat shock cognate 71 kDa protein                                                                      | HSPA8   |
| 166 | + | 4.685 | 2.187 | Q9Y2L1 | Exosome complex exonuclease RRP44                                                                      | DIS3    |
| 167 | + | 1.612 | 2.184 | P39748 | Flap endonuclease 1                                                                                    | FEN1    |
| 168 | + | 3.908 | 2.164 | P20042 | Eukaryotic translation initiation factor 2 subunit 2                                                   | EIF2S2  |
| 169 | + | 5.043 | 2.149 | P48643 | T-complex protein 1 subunit epsilon                                                                    | CCT5    |
| 170 | + | 1.575 | 2.149 | P24534 | Elongation factor 1-beta                                                                               | EEF1B2  |
| 171 | + | 3.405 | 2.145 | O75534 | Cold shock domain-containing protein E1                                                                | CSDE1   |
| 172 | + | 2.926 | 2.141 | Q13151 | Heterogeneous nuclear ribonucleoprotein A0                                                             | HNRNPA0 |
| 173 | + | 2.281 | 2.139 | P43246 | DNA mismatch repair protein Msh2                                                                       | MSH2    |
| 174 | + | 4.241 | 2.122 | P50395 | Rab GDP dissociation inhibitor beta                                                                    | GDI2    |
| 175 | + | 3.244 | 2.110 | Q9Y2Z0 | Suppressor of G2 allele of SKP1 homolog                                                                | SUGT1   |

## Identified Proteins

|     |   |       |       |              |                                                                                                                           |                 |
|-----|---|-------|-------|--------------|---------------------------------------------------------------------------------------------------------------------------|-----------------|
| 176 | + | 2.649 | 2.091 | Q13838       | Spliceosome RNA helicase DDX39B                                                                                           | DDX39B          |
| 177 | + | 2.895 | 2.085 | Q9Y6E2       | Basic leucine zipper and W2 domain-containing protein 2                                                                   | BZW2            |
| 178 | + | 4.894 | 2.080 | Q9BWD1       | Acetyl-CoA acetyltransferase, cytosolic                                                                                   | ACAT2           |
| 179 | + | 3.592 | 2.079 | P60842       | Eukaryotic initiation factor 4A-I                                                                                         | EIF4A1          |
| 180 | + | 3.954 | 2.068 | P50990       | T-complex protein 1 subunit theta                                                                                         | CCT8            |
| 181 | + | 3.032 | 2.062 | Q00610       | Clahtin heavy chain 1                                                                                                     | CLTC            |
| 182 | + | 6.519 | 2.053 | P18669       | Phosphoglycerate mutase 1                                                                                                 | PGAM1           |
| 183 | + | 1.319 | 2.036 | P04181       | Ornithine aminotransferase, mitochondrial;Ornithine aminotransferase, hepatic form;Ornithine aminotransferase, renal form | OAT             |
| 184 | + | 2.557 | 2.022 | O00154       | Cytosolic acyl coenzyme A thioester hydrolase                                                                             | ACOT7           |
| 185 | + | 1.493 | 2.021 | P23246       | Splicing factor, proline- and glutamine-rich                                                                              | SFPQ            |
| 186 | + | 6.406 | 2.002 | P49327       | Fatty acid synthase;[Acyl-carrier-protein] S-acetyltransferase;[Acyl-carrier-protein] S-malonyltransferase                | FASN            |
| 187 | + | 4.397 | 1.996 | O00299       | Chloride intracellular channel protein 1                                                                                  | CLIC1           |
| 188 | + | 3.250 | 1.984 | P55072       | Transitional endoplasmic reticulum ATPase                                                                                 | VCP             |
| 189 | + | 3.955 | 1.969 | P62258       | 14-3-3 protein epsilon                                                                                                    | YWHAE           |
| 190 | + | 2.036 | 1.960 | P55209       | Nucleosome assembly protein 1-like 1                                                                                      | NAP1L1          |
| 191 | + | 2.194 | 1.935 | Q8TEX9       | Importin-4                                                                                                                | IPO4            |
| 192 | + | 1.951 | 1.934 | P07910;P0DMR | Heterogeneous nuclear ribonucleoproteins C1/C2;Heterogeneous nuclear ribonucleoprotein C-like 4                           | HNRNPC;HNRNPCL4 |
| 193 | + | 1.835 | 1.930 | P55010       | Eukaryotic translation initiation factor 5                                                                                | EIF5            |
| 194 | + | 4.505 | 1.916 | O14929       | Histone acetyltransferase type B catalytic subunit                                                                        | HAT1            |
| 195 | + | 4.094 | 1.915 | Q13263       | Transcription intermediary factor 1-beta                                                                                  | TRIM28          |
| 196 | + | 2.432 | 1.910 | Q00341       | Vigilin                                                                                                                   | HDLBP           |
| 197 | + | 1.874 | 1.905 | P22061       | Protein-L-isoaspartate(D-aspartate) O-methyltransferase                                                                   | PCMT1           |
| 198 | + | 1.554 | 1.904 | O75643       | U5 small nuclear ribonucleoprotein 200 kDa helicase                                                                       | SNRNP200        |
| 199 | + | 2.091 | 1.904 | Q9NUU7       | ATP-dependent RNA helicase DDX19A                                                                                         | DDX19A          |
| 200 | + | 3.250 | 1.892 | Q7Z6Z7       | E3 ubiquitin-protein ligase HUWE1                                                                                         | HUWE1           |
| 201 | + | 3.944 | 1.886 | P60981       | Destrin                                                                                                                   | DSTN            |
| 202 | + | 2.047 | 1.886 | P60174       | Triosephosphate isomerase                                                                                                 | TPI1            |

## Appendix

|     |   |       |       |        |                                                                   |         |
|-----|---|-------|-------|--------|-------------------------------------------------------------------|---------|
| 203 | + | 2.520 | 1.884 | P51991 | Heterogeneous nuclear ribonucleoprotein A3                        | HNRNPA3 |
| 204 | + | 3.115 | 1.880 | O60841 | Eukaryotic translation initiation factor 5B                       | EIF5B   |
| 205 | + | 3.996 | 1.880 | P49419 | Alpha-aminoacidic semialdehyde dehydrogenase                      | ALDH7A1 |
| 206 | + | 1.401 | 1.878 | P26639 | Threonine--tRNA ligase, cytoplasmic                               | TARS    |
| 207 | + | 2.138 | 1.873 | P23526 | Adenosylhomocysteinase                                            | AHCY    |
| 208 | + | 4.018 | 1.871 | P23528 | Cofilin-1                                                         | CFL1    |
| 209 | + | 2.081 | 1.868 | Q12905 | Interleukin enhancer-binding factor 2                             | ILF2    |
| 210 | + | 2.990 | 1.857 | O00267 | Transcription elongation factor SPT5                              | SUPT5H  |
| 211 | + | 3.178 | 1.851 | Q86VP6 | Cullin-associated NEDD8-dissociated protein 1                     | CAND1   |
| 212 | + | 1.599 | 1.850 | P12270 | Nucleoprotein TPR                                                 | TPR     |
| 213 | + | 4.320 | 1.847 | O43684 | Mitotic checkpoint protein BUB3                                   | BUB3    |
| 214 | + | 2.071 | 1.847 | P06576 | ATP synthase subunit beta, mitochondrial                          | ATP5B   |
| 215 | + | 4.341 | 1.836 | P15170 | Eukaryotic peptide chain release factor GTP-binding subunit ERF3A | GSPT1   |
| 216 | + | 2.423 | 1.833 | P52209 | 6-phosphogluconate dehydrogenase, decarboxylating                 | PGD     |
| 217 | + | 3.515 | 1.815 | Q15645 | Pachytene checkpoint protein 2 homolog                            | TRIP13  |
| 218 | + | 3.006 | 1.812 | Q9NPD8 | Ubiquitin-conjugating enzyme E2 T                                 | UBE2T   |
| 219 | + | 1.666 | 1.810 | P49591 | Serine--tRNA ligase, cytoplasmic                                  | SARS    |
| 220 | + | 3.535 | 1.798 | Q9Y262 | Eukaryotic translation initiation factor 3 subunit L              | EIF3L   |
| 221 | + | 4.912 | 1.795 | Q14152 | Eukaryotic translation initiation factor 3 subunit A              | EIF3A   |
| 222 | + | 1.884 | 1.765 | O75390 | Citrate synthase, mitochondrial                                   | CS      |
| 223 | + | 6.756 | 1.753 | P43897 | Elongation factor Ts, mitochondrial                               | TSFM    |
| 224 | + | 1.653 | 1.737 | P15531 | Nucleoside diphosphate kinase A                                   | NME1    |
| 225 | + | 2.555 | 1.730 | P60228 | Eukaryotic translation initiation factor 3 subunit E              | EIF3E   |
| 226 | + | 1.928 | 1.722 | O00148 | ATP-dependent RNA helicase DDX39A                                 | DDX39A  |
| 227 | + | 4.624 | 1.721 | P54578 | Ubiquitin carboxyl-terminal hydrolase 14                          | USP14   |
| 228 | + | 1.493 | 1.657 | Q15393 | Splicing factor 3B subunit 3                                      | SF3B3   |
| 229 | + | 1.678 | 1.656 | P07741 | Adenine phosphoribosyltransferase                                 | APRT    |
| 230 | + | 3.320 | 1.655 | P63104 | 14-3-3 protein zeta/delta                                         | YWHAZ   |
| 231 | + | 1.500 | 1.651 | Q14257 | Reticulocalbin-2                                                  | RCN2    |

|     |   |       |       |               |                                                                                                                        |              |
|-----|---|-------|-------|---------------|------------------------------------------------------------------------------------------------------------------------|--------------|
| 232 | + | 3.116 | 1.644 | Q13642        | Four and a half LIM domains protein 1                                                                                  | FHL1         |
| 233 | + | 3.410 | 1.644 | P07737        | Profilin-1                                                                                                             | PFN1         |
| 234 | + | 6.015 | 1.633 | Q00839        | Heterogeneous nuclear ribonucleoprotein U                                                                              | HNRNPU       |
| 235 | + | 3.501 | 1.631 | Q13907        | Isopentenyl-diphosphate Delta-isomerase 1                                                                              | ID1I         |
| 236 | + | 1.654 | 1.616 | P62851        | 40S ribosomal protein S25                                                                                              | RPS25        |
| 237 | + | 3.433 | 1.612 | O43175        | D-3-phosphoglycerate dehydrogenase                                                                                     | PHGDH        |
| 238 | + | 2.441 | 1.595 | Q16630        | Cleavage and polyadenylation specificity factor subunit 6                                                              | CPSF6        |
| 239 | + | 4.008 | 1.554 | Q6P2E9        | Enhancer of mRNA-decapping protein 4                                                                                   | EDC4         |
| 240 | + | 3.849 | 1.526 | Q5VYK3        | Proteasome-associated protein ECM29 homolog                                                                            | ECM29        |
| 241 | + | 2.221 | 1.525 | Q9Y295        | Developmentally-regulated GTP-binding protein 1                                                                        | DRG1         |
| 242 | + | 3.411 | 1.507 | P62826        | GTP-binding nuclear protein Ran                                                                                        | RAN          |
| 243 | + | 3.031 | 1.490 | P11802        | Cyclin-dependent kinase 4                                                                                              | CDK4         |
| 244 | + | 3.649 | 1.483 | Q9NZI8        | Insulin-like growth factor 2 mRNA-binding protein 1                                                                    | IGF2BP1      |
| 245 | + | 4.774 | 1.468 | P61978        | Heterogeneous nuclear ribonucleoprotein K                                                                              | HNRNPK       |
| 246 | + | 3.734 | 1.459 | P62191        | 26S protease regulatory subunit 4                                                                                      | PSMC1        |
| 247 | + | 4.359 | 1.417 | Q99613;B5ME19 | Eukaryotic translation initiation factor 3 subunit C;Eukaryotic translation initiation factor 3 subunit C-like protein | EIF3C;EIF3CL |
| 248 | + | 5.306 | 1.415 | Q9NRN7        | L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase                                              | AASDHPPT     |
| 249 | + | 4.460 | 1.387 | Q08211        | ATP-dependent RNA helicase A                                                                                           | DHX9         |

2.3 Proteins Significantly Enriched in the Heat Experiment in Comparison to the Beads Control

Table 9: Proteins significantly enriched in the heat experiment in comparison to the beads control.

|   | Significant | -log p | Difference | Majority protein IDs | Protein names                   | Gene names |
|---|-------------|--------|------------|----------------------|---------------------------------|------------|
| 1 | +           | 8.390  | 7.613      | P15531               | Nucleoside diphosphate kinase A | NME1       |
| 2 | +           | 6.008  | 7.144      | P06733               | Alpha-enolase                   | ENO1       |
| 3 | +           | 8.418  | 6.844      | P60174               | Triosephosphate isomerase       | TPI1       |
| 4 | +           | 8.592  | 6.844      | P00338               | L-lactate dehydrogenase A chain | LDHA       |

## Appendix

|    |   |        |       |        |                                                                                                                                           |        |
|----|---|--------|-------|--------|-------------------------------------------------------------------------------------------------------------------------------------------|--------|
| 5  | + | 8.226  | 6.568 | P04075 | Fructose-bisphosphate aldolase A                                                                                                          | ALDOA  |
| 6  | + | 10.679 | 6.340 | P63244 | Guanine nucleotide-binding protein subunit beta-2-like 1;Guanine nucleotide-binding protein subunit beta-2-like 1, N-terminally processed | GNB2L1 |
| 7  | + | 7.208  | 6.298 | P07195 | L-lactate dehydrogenase B chain                                                                                                           | LDB    |
| 8  | + | 9.637  | 6.298 | P30086 | Phosphatidylethanolamine-binding protein 1;Hippocampal cholinergic neurostimulating peptide                                               | PEBP1  |
| 9  | + | 10.956 | 6.274 | P18669 | Phosphoglycerate mutase 1                                                                                                                 | PGAM1  |
| 10 | + | 6.850  | 6.154 | P12277 | Creatine kinase B-type                                                                                                                    | CKB    |
| 11 | + | 7.025  | 6.040 | P08865 | 40S ribosomal protein SA                                                                                                                  | RPSA   |
| 12 | + | 7.541  | 5.519 | P27348 | 14-3-3 protein theta                                                                                                                      | YWHAQ  |
| 13 | + | 6.270  | 5.475 | P00558 | Phosphoglycerate kinase 1                                                                                                                 | PGK1   |
| 14 | + | 6.890  | 5.236 | Q15185 | Prostaglandin H synthase 3                                                                                                                | PTGES3 |
| 15 | + | 3.916  | 5.236 | P37802 | Transgelin-2                                                                                                                              | TAGLN2 |
| 16 | + | 8.900  | 5.214 | P62258 | 14-3-3 protein epsilon                                                                                                                    | YWHAE  |
| 17 | + | 8.330  | 5.211 | P55072 | Transitional endoplasmic reticulum ATPase                                                                                                 | VCP    |
| 18 | + | 6.698  | 5.104 | Q92598 | Heat shock protein 105 kDa                                                                                                                | HSPH1  |
| 19 | + | 7.227  | 5.099 | Q15181 | Inorganic pyrophosphatase                                                                                                                 | PPA1   |
| 20 | + | 8.240  | 5.001 | P00505 | Aspartate aminotransferase, mitochondrial                                                                                                 | GOT2   |
| 21 | + | 6.617  | 4.982 | P23526 | Adenosylhomocysteinase                                                                                                                    | AHCY   |
| 22 | + | 12.589 | 4.946 | P04406 | Glyceraldehyde-3-phosphate dehydrogenase                                                                                                  | GAPDH  |
| 23 | + | 7.414  | 4.894 | P29401 | Transketolase                                                                                                                             | TKT    |
| 24 | + | 9.122  | 4.867 | P40925 | Malate dehydrogenase, cytoplasmic                                                                                                         | MDH1   |
| 25 | + | 7.503  | 4.860 | P34932 | Heat shock 70 kDa protein 4                                                                                                               | HSPA4  |
| 26 | + | 6.906  | 4.855 | P16152 | Carbonyl reductase [NADPH] 1                                                                                                              | CBR1   |
| 27 | + | 6.090  | 4.795 | P27797 | Calreticulin                                                                                                                              | CALR   |
| 28 | + | 11.736 | 4.783 | P00918 | Carbonic anhydrase 2                                                                                                                      | CA2    |
| 29 | + | 5.280  | 4.756 | P40926 | Malate dehydrogenase, mitochondrial                                                                                                       | MDH2   |
| 30 | + | 7.409  | 4.748 | P29692 | Elongation factor 1-delta                                                                                                                 | EEF1D  |
| 31 | + | 5.392  | 4.714 | P22234 | Multifunctional protein ADE2;Phosphoribosylaminoimidazole-succinocarboxamide synthase;Phosphoribosylaminoimidazole carboxylase            | PAICS  |
| 32 | + | 7.993  | 4.689 | P63104 | 14-3-3 protein zeta/delta                                                                                                                 | YWHAZ  |

## Identified Proteins

|    |   |       |       |               |                                                                                                                            |               |
|----|---|-------|-------|---------------|----------------------------------------------------------------------------------------------------------------------------|---------------|
| 33 | + | 7.442 | 4.643 | P60891        | Ribose-phosphate pyrophosphokinase 1                                                                                       | PRPS1         |
| 34 | + | 4.101 | 4.624 | Q13162        | Peroxioredoxin-4                                                                                                           | PRDX4         |
| 35 | + | 5.956 | 4.561 | P46783;Q9NQ39 | 40S ribosomal protein S10;Putative 40S ribosomal protein S10-like                                                          | RPS10;RPS10P5 |
| 36 | + | 5.339 | 4.527 | Q09028        | Histone-binding protein RBBP4                                                                                              | RBBP4         |
| 37 | + | 7.364 | 4.497 | Q99873        | Protein arginine N-methyltransferase 1                                                                                     | PRMT1         |
| 38 | + | 8.608 | 4.385 | P31939        | Bifunctional purine biosynthesis protein PURH;Phosphoribosylaminoimidazolecarboxamide formyltransferase;IMP cyclohydrolase | ATIC          |
| 39 | + | 5.303 | 4.379 | Q07021        | Complement component 1 Q subcomponent-binding protein, mitochondrial                                                       | C1QBP         |
| 40 | + | 8.263 | 4.370 | Q9Y3F4        | Serine-threonine kinase receptor-associated protein                                                                        | STRAP         |
| 41 | + | 7.080 | 4.327 | P06744        | Glucose-6-phosphate isomerase                                                                                              | GPI           |
| 42 | + | 5.666 | 4.299 | P12268        | Inosine-5-monophosphate dehydrogenase 2                                                                                    | IMPDH2        |
| 43 | + | 5.917 | 4.298 | Q15084        | Protein disulfide-isomerase A6                                                                                             | PDI A6        |
| 44 | + | 6.307 | 4.291 | Q14166        | Tubulin--tyrosine ligase-like protein 12                                                                                   | TTL12         |
| 45 | + | 9.001 | 4.284 | P31948        | Stress-induced-phosphoprotein 1                                                                                            | STIP1         |
| 46 | + | 7.360 | 4.252 | Q04760        | Lactoylglutathione lyase                                                                                                   | GLO1          |
| 47 | + | 2.361 | 4.230 | P14174        | Macrophage migration inhibitory factor                                                                                     | MIF           |
| 48 | + | 4.686 | 4.123 | Q9HB71        | Calcyclin-binding protein                                                                                                  | CACYBP        |
| 49 | + | 7.573 | 4.064 | P52565        | Rho GDP-dissociation inhibitor 1                                                                                           | ARHGDI A      |
| 50 | + | 7.968 | 4.063 | P10768        | S-formylglutathione hydrolase                                                                                              | ESD           |
| 51 | + | 6.340 | 4.047 | P12004        | Proliferating cell nuclear antigen                                                                                         | PCNA          |
| 52 | + | 7.071 | 4.039 | P33993        | DNA replication licensing factor MCM7                                                                                      | MCM7          |
| 53 | + | 7.895 | 4.026 | P55209        | Nucleosome assembly protein 1-like 1                                                                                       | NAP1L1        |
| 54 | + | 8.358 | 3.986 | O43390        | Heterogeneous nuclear ribonucleoprotein R                                                                                  | HNRNPR        |
| 55 | + | 7.824 | 3.938 | P07237        | Protein disulfide-isomerase                                                                                                | P4HB          |
| 56 | + | 6.325 | 3.922 | P26641        | Elongation factor 1-gamma                                                                                                  | EEF1G         |
| 57 | + | 7.659 | 3.908 | O75390        | Citrate synthase, mitochondrial                                                                                            | CS            |
| 58 | + | 7.628 | 3.853 | P24534        | Elongation factor 1-beta                                                                                                   | EEF1B2        |
| 59 | + | 5.694 | 3.806 | P22626        | Heterogeneous nuclear ribonucleoproteins A2/B1                                                                             | HNRNPA2B1     |
| 60 | + | 5.640 | 3.803 | Q14103        | Heterogeneous nuclear ribonucleoprotein D0                                                                                 | HNRNPD        |

## Appendix

|    |   |       |       |               |                                                                                                             |            |
|----|---|-------|-------|---------------|-------------------------------------------------------------------------------------------------------------|------------|
| 61 | + | 5.967 | 3.773 | P11021        | 78 kDa glucose-regulated protein                                                                            | HSPA5      |
| 62 | + | 3.527 | 3.772 | Q02790        | Peptidyl-prolyl cis-trans isomerase FKBP4;Peptidyl-prolyl cis-trans isomerase FKBP4, N-terminally processed | FKBP4      |
| 63 | + | 5.187 | 3.752 | Q01105;P0DMEO | Protein SET;Protein SETSIP                                                                                  | SET;SETSIP |
| 64 | + | 6.230 | 3.729 | P49720        | Proteasome subunit beta type-3                                                                              | PSMB3      |
| 65 | + | 4.923 | 3.704 | Q9UHD1        | Cysteine and histidine-rich domain-containing protein 1                                                     | CHORDC1    |
| 66 | + | 7.071 | 3.698 | P55786        | Puromycin-sensitive aminopeptidase                                                                          | NPEPPS     |
| 67 | + | 4.149 | 3.664 | P13797        | Plastin-3                                                                                                   | PLS3       |
| 68 | + | 4.285 | 3.659 | P30048        | Thioredoxin-dependent peroxide reductase, mitochondrial                                                     | PRDX3      |
| 69 | + | 7.324 | 3.613 | Q16543        | Hsp90 co-chaperone Cdc37;Hsp90 co-chaperone Cdc37, N-terminally processed                                   | CDC37      |
| 70 | + | 5.663 | 3.578 | P62495        | Eukaryotic peptide chain release factor subunit 1                                                           | ETF1       |
| 71 | + | 4.418 | 3.554 | P38646        | Stress-70 protein, mitochondrial                                                                            | HSPA9      |
| 72 | + | 7.118 | 3.531 | P62136        | Serine/threonine-protein phosphatase PP1-alpha catalytic subunit                                            | PPP1CA     |
| 73 | + | 8.740 | 3.519 | P30101        | Protein disulfide-isomerase A3                                                                              | PDI A3     |
| 74 | + | 6.151 | 3.516 | O00299        | Chloride intracellular channel protein 1                                                                    | CLIC1      |
| 75 | + | 4.724 | 3.511 | P07900        | Heat shock protein HSP 90-alpha                                                                             | HSP90AA1   |
| 76 | + | 8.043 | 3.510 | Q9UQ80        | Proliferation-associated protein 2G4                                                                        | PA2G4      |
| 77 | + | 5.456 | 3.495 | P52209        | 6-phosphogluconate dehydrogenase, decarboxylating                                                           | PGD        |
| 78 | + | 4.081 | 3.478 | P23396        | 40S ribosomal protein S3                                                                                    | RPS3       |
| 79 | + | 4.611 | 3.437 | O95433        | Activator of 90 kDa heat shock protein ATPase homolog 1                                                     | AHSA1      |
| 80 | + | 7.841 | 3.419 | P56192        | Methionine--tRNA ligase, cytoplasmic                                                                        | MARS       |
| 81 | + | 3.450 | 3.416 | P11142        | Heat shock cognate 71 kDa protein                                                                           | HSPA8      |
| 82 | + | 5.839 | 3.397 | O75832        | 26S proteasome non-ATPase regulatory subunit 10                                                             | PSMD10     |
| 83 | + | 4.680 | 3.391 | P14625        | Endoplasmic                                                                                                 | HSP90B1    |
| 84 | + | 6.640 | 3.390 | P55884        | Eukaryotic translation initiation factor 3 subunit B                                                        | EIF3B      |
| 85 | + | 6.117 | 3.376 | P31153        | S-adenosylmethionine synthase isoform type-2                                                                | MAT2A      |
| 86 | + | 3.683 | 3.365 | P22314        | Ubiquitin-like modifier-activating enzyme 1                                                                 | UBA1       |
| 87 | + | 6.255 | 3.322 | P26639        | Threonine--tRNA ligase, cytoplasmic                                                                         | TARS       |
| 88 | + | 6.156 | 3.309 | P17980        | 26S protease regulatory subunit 6A                                                                          | PSMC3      |

## Identified Proteins

|     |   |       |       |               |                                                                                                                                                                                                                                                    |                   |
|-----|---|-------|-------|---------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| 89  | + | 5.818 | 3.287 | P23284        | Peptidyl-prolyl cis-trans isomerase B                                                                                                                                                                                                              | PIIB              |
| 90  | + | 3.638 | 3.269 | P07910;PODMRI | Heterogeneous nuclear ribonucleoproteins C1/C2;Heterogeneous nuclear ribonucleoprotein C-like 4;Heterogeneous nuclear ribonucleoprotein C-like 1;Heterogeneous nuclear ribonucleoprotein C-like 3;Heterogeneous nuclear ribonucleoprotein C-like 2 | HNRNPC;HNRNPCL4   |
| 91  | + | 3.936 | 3.215 | P54136        | Arginine--tRNA ligase, cytoplasmic                                                                                                                                                                                                                 | RARS              |
| 92  | + | 5.371 | 3.191 | Q9P2J5        | Leucine--tRNA ligase, cytoplasmic                                                                                                                                                                                                                  | LARS              |
| 93  | + | 5.832 | 3.172 | P35637;Q92804 | RNA-binding protein FUS;TATA-binding protein-associated factor 2N                                                                                                                                                                                  | FUS;TAF15         |
| 94  | + | 4.708 | 3.172 | Q9Y265        | RuvB-like 1                                                                                                                                                                                                                                        | RUVBL1            |
| 95  | + | 4.474 | 3.148 | P53396        | ATP-citrate synthase                                                                                                                                                                                                                               | ACLY              |
| 96  | + | 5.055 | 3.143 | P09651;Q32P51 | Heterogeneous nuclear ribonucleoprotein A1;Heterogeneous nuclear ribonucleoprotein A1, N-terminally processed;Heterogeneous nuclear ribonucleoprotein A1-like 2                                                                                    | HNRNPA1;HNRNPA1L2 |
| 97  | + | 6.300 | 3.125 | P13489        | Ribonuclease inhibitor                                                                                                                                                                                                                             | RNH1              |
| 98  | + | 3.620 | 3.105 | P32119        | Peroxioredoxin-2                                                                                                                                                                                                                                   | PRDX2             |
| 99  | + | 4.318 | 3.105 | P06493        | Cyclin-dependent kinase 1                                                                                                                                                                                                                          | CDK1              |
| 100 | + | 5.337 | 3.076 | P49588        | Alanine--tRNA ligase, cytoplasmic                                                                                                                                                                                                                  | AARS              |
| 101 | + | 8.030 | 3.072 | P50395        | Rab GDP dissociation inhibitor beta                                                                                                                                                                                                                | GDI2              |
| 102 | + | 7.501 | 3.068 | P09960        | Leukotriene A-4 hydrolase                                                                                                                                                                                                                          | LTA4H             |
| 103 | + | 6.156 | 3.045 | P27695        | DNA-(apurinic or apyrimidinic site) lyase;DNA-(apurinic or apyrimidinic site) lyase, mitochondrial                                                                                                                                                 | APEX1             |
| 104 | + | 2.635 | 2.941 | P06748        | Nucleophosmin                                                                                                                                                                                                                                      | NPM1              |
| 105 | + | 5.292 | 2.929 | P22392;O60361 | Nucleoside diphosphate kinase B;Putative nucleoside diphosphate kinase                                                                                                                                                                             | NME2;NME2P1       |
| 106 | + | 3.635 | 2.928 | P78417        | Glutathione S-transferase omega-1                                                                                                                                                                                                                  | GSTO1             |
| 107 | + | 3.680 | 2.922 | P18124        | 60S ribosomal protein L7                                                                                                                                                                                                                           | RPL7              |
| 108 | + | 6.208 | 2.912 | P18754        | Regulator of chromosome condensation                                                                                                                                                                                                               | RCC1              |
| 109 | + | 5.537 | 2.903 | P62826        | GTP-binding nuclear protein Ran                                                                                                                                                                                                                    | RAN               |
| 110 | + | 7.648 | 2.902 | P07737        | Profilin-1                                                                                                                                                                                                                                         | PFN1              |
| 111 | + | 6.314 | 2.889 | P61981        | 14-3-3 protein gamma;14-3-3 protein gamma, N-terminally processed                                                                                                                                                                                  | YWHAH             |
| 112 | + | 5.329 | 2.881 | Q9UBQ5        | Eukaryotic translation initiation factor 3 subunit K                                                                                                                                                                                               | EIF3K             |
| 113 | + | 6.283 | 2.881 | P28074        | Proteasome subunit beta type-5                                                                                                                                                                                                                     | PSMB5             |
| 114 | + | 5.450 | 2.844 | P67775;P62714 | Serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform;Serine/threonine-protein phosphatase 2A catalytic subunit beta isoform                                                                                                     | PPP2CA;PPP2CB     |
| 115 | + | 6.620 | 2.829 | P23381        | Tryptophan--tRNA ligase, cytoplasmic;T1-TrpRS;T2-TrpRS                                                                                                                                                                                             | WARS              |

## Appendix

|     |   |       |       |        |                                                                             |         |
|-----|---|-------|-------|--------|-----------------------------------------------------------------------------|---------|
| 116 | + | 3.860 | 2.819 | Q13200 | 26S proteasome non-ATPase regulatory subunit 2                              | PSMD2   |
| 117 | + | 6.326 | 2.799 | P49591 | Serine--tRNA ligase, cytoplasmic                                            | SARS    |
| 118 | + | 5.159 | 2.798 | P26038 | Moesin                                                                      | MSN     |
| 119 | + | 6.723 | 2.798 | P50990 | T-complex protein 1 subunit theta                                           | CCT8    |
| 120 | + | 1.897 | 2.793 | P49915 | GMP synthase [glutamine-hydrolyzing]                                        | GMPS    |
| 121 | + | 5.431 | 2.792 | P60981 | Destrin                                                                     | DSTN    |
| 122 | + | 5.753 | 2.791 | P26640 | Valine--tRNA ligase                                                         | VARS    |
| 123 | + | 2.942 | 2.765 | Q12905 | Interleukin enhancer-binding factor 2                                       | ILF2    |
| 124 | + | 5.320 | 2.755 | Q9Y617 | Phosphoserine aminotransferase                                              | PSAT1   |
| 125 | + | 7.810 | 2.735 | P63261 | Actin, cytoplasmic 2;Actin, cytoplasmic 2, N-terminally processed           | ACTG1   |
| 126 | + | 6.238 | 2.729 | Q13867 | Bleomycin hydrolase                                                         | BLMH    |
| 127 | + | 3.939 | 2.711 | Q9UMS4 | Pre-mRNA-processing factor 19                                               | PRPF19  |
| 128 | + | 2.590 | 2.710 | P14866 | Heterogeneous nuclear ribonucleoprotein L                                   | HNRNPL  |
| 129 | + | 5.075 | 2.706 | P12532 | Creatine kinase U-type, mitochondrial                                       | CKMT1A  |
| 130 | + | 4.205 | 2.704 | Q9Y262 | Eukaryotic translation initiation factor 3 subunit L                        | EIF3L   |
| 131 | + | 5.171 | 2.700 | P31946 | 14-3-3 protein beta/alpha;14-3-3 protein beta/alpha, N-terminally processed | YWHA8   |
| 132 | + | 3.357 | 2.646 | P30050 | 60S ribosomal protein L12                                                   | RPL12   |
| 133 | + | 5.774 | 2.638 | P41252 | Isoleucine--tRNA ligase, cytoplasmic                                        | IARS    |
| 134 | + | 6.963 | 2.619 | O76003 | Glutaredoxin-3                                                              | GLRX3   |
| 135 | + | 5.105 | 2.609 | O60506 | Heterogeneous nuclear ribonucleoprotein Q                                   | SYNCRIP |
| 136 | + | 8.843 | 2.591 | P14618 | Pyruvate kinase PKM                                                         | PKM     |
| 137 | + | 3.962 | 2.576 | P46777 | 60S ribosomal protein L5                                                    | RPL5    |
| 138 | + | 8.817 | 2.563 | P23528 | Cofilin-1                                                                   | CFL1    |
| 139 | + | 3.783 | 2.542 | Q15056 | Eukaryotic translation initiation factor 4H                                 | EIF4H   |
| 140 | + | 7.575 | 2.540 | O43684 | Mitotic checkpoint protein BUB3                                             | BUB3    |
| 141 | + | 4.294 | 2.533 | Q00796 | Sorbitol dehydrogenase                                                      | SORD    |
| 142 | + | 5.587 | 2.484 | Q15691 | Microtubule-associated protein RP/EB family member 1                        | MAPRE1  |
| 143 | + | 3.428 | 2.473 | Q02543 | 60S ribosomal protein L18a                                                  | RPL18A  |
| 144 | + | 4.194 | 2.464 | P55735 | Protein SEC13 homolog                                                       | SEC13   |

## Identified Proteins

|     |   |       |       |               |                                                                                                                                                                                    |               |
|-----|---|-------|-------|---------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| 145 | + | 4.531 | 2.464 | P12814        | Alpha-actinin-1                                                                                                                                                                    | ACTN1         |
| 146 | + | 3.211 | 2.458 | P33992        | DNA replication licensing factor MCM5                                                                                                                                              | MCM5          |
| 147 | + | 4.706 | 2.458 | Q9GZS3        | WD repeat-containing protein 61;WD repeat-containing protein 61, N-terminally processed                                                                                            | WDR61         |
| 148 | + | 6.680 | 2.450 | Q13907        | Isopenentenyl-diphosphate Delta-isomerase 1                                                                                                                                        | IDI1          |
| 149 | + | 2.939 | 2.449 | P47897        | Glutamine--tRNA ligase                                                                                                                                                             | QARS          |
| 150 | + | 6.268 | 2.447 | P07814        | Bifunctional glutamate/proline--tRNA ligase;Glutamate--tRNA ligase;Proline--tRNA ligase                                                                                            | EPRS          |
| 151 | + | 6.395 | 2.442 | P22102        | Trifunctional purine biosynthetic protein adenosine-3;Phosphoribosylamine--glycine ligase;Phosphoribosylformylglycinamide cyclo-ligase;Phosphoribosylglycinamide formyltransferase | GART          |
| 152 | + | 5.758 | 2.435 | P30046;A6NHG4 | D-dopachrome decarboxylase;D-dopachrome decarboxylase-like protein                                                                                                                 | DDT;DDTL      |
| 153 | + | 2.963 | 2.426 | O43776        | Asparagine--tRNA ligase, cytoplasmic                                                                                                                                               | NARS          |
| 154 | + | 3.339 | 2.416 | P25205        | DNA replication licensing factor MCM3                                                                                                                                              | MCM3          |
| 155 | + | 3.764 | 2.389 | Q9P258        | Protein RCC2                                                                                                                                                                       | RCC2          |
| 156 | + | 5.080 | 2.388 | O75821        | Eukaryotic translation initiation factor 3 subunit G                                                                                                                               | EIF3G         |
| 157 | + | 4.086 | 2.383 | P25398        | 40S ribosomal protein S12                                                                                                                                                          | RPS12         |
| 158 | + | 2.594 | 2.378 | Q14697        | Neutral alpha-glucosidase AB                                                                                                                                                       | GANAB         |
| 159 | + | 7.339 | 2.360 | P11766        | Alcohol dehydrogenase class-3                                                                                                                                                      | ADH5          |
| 160 | + | 3.175 | 2.351 | P08243        | Asparagine synthetase [glutamine-hydrolyzing]                                                                                                                                      | ASNS          |
| 161 | + | 4.653 | 2.350 | P62249        | 40S ribosomal protein S16                                                                                                                                                          | RPS16         |
| 162 | + | 4.768 | 2.346 | P13667        | Protein disulfide-isomerase A4                                                                                                                                                     | PDI4          |
| 163 | + | 4.187 | 2.345 | P05455        | Lupus La protein                                                                                                                                                                   | SSB           |
| 164 | + | 2.904 | 2.332 | P07741        | Adenine phosphoribosyltransferase                                                                                                                                                  | APRT          |
| 165 | + | 4.956 | 2.290 | P42771;P42772 | Cyclin-dependent kinase inhibitor 2A;Cyclin-dependent kinase 4 inhibitor B                                                                                                         | CDKN2A;CDKN2B |
| 166 | + | 4.796 | 2.286 | P60866        | 40S ribosomal protein S20                                                                                                                                                          | RPS20         |
| 167 | + | 3.552 | 2.284 | P33316        | Deoxyuridine 5-triphosphate nucleotidohydrolase, mitochondrial                                                                                                                     | DUT           |
| 168 | + | 2.896 | 2.275 | P54577        | Tyrosine--tRNA ligase, cytoplasmic;Tyrosine--tRNA ligase, cytoplasmic, N-terminally processed                                                                                      | YARS          |
| 169 | + | 5.359 | 2.274 | P04818        | Thymidylate synthase                                                                                                                                                               | TYMS          |
| 170 | + | 5.034 | 2.262 | P61158        | Actin-related protein 3                                                                                                                                                            | ACTR3         |
| 171 | + | 6.076 | 2.256 | P49411        | Elongation factor Tu, mitochondrial                                                                                                                                                | TUFM          |
| 172 | + | 4.637 | 2.250 | Q06830        | Peroxisedoxin-1                                                                                                                                                                    | PRDX1         |

## Appendix

|     |   |       |       |               |                                                                                                     |               |
|-----|---|-------|-------|---------------|-----------------------------------------------------------------------------------------------------|---------------|
| 173 | + | 4.342 | 2.245 | Q14566        | DNA replication licensing factor MCM6                                                               | MCM6          |
| 174 | + | 6.018 | 2.237 | P39687        | Acidic leucine-rich nuclear phosphoprotein 32 family member A                                       | ANP32A        |
| 175 | + | 2.562 | 2.222 | P61247        | 40S ribosomal protein S3a                                                                           | RPS3A         |
| 176 | + | 5.261 | 2.216 | P62937        | Peptidyl-prolyl cis-trans isomerase A;Peptidyl-prolyl cis-trans isomerase A, N-terminally processed | PIPA          |
| 177 | + | 1.544 | 2.215 | Q12906        | Interleukin enhancer-binding factor 3                                                               | ILF3          |
| 178 | + | 3.974 | 2.208 | P62837;P61077 | Ubiquitin-conjugating enzyme E2 D2;Ubiquitin-conjugating enzyme E2 D3                               | UBE2D2;UBE2D3 |
| 179 | + | 5.680 | 2.197 | Q9Y266        | Nuclear migration protein nudC                                                                      | NUDC          |
| 180 | + | 2.595 | 2.193 | P62888        | 60S ribosomal protein L30                                                                           | RPL30         |
| 181 | + | 2.666 | 2.191 | P61081        | NEDD8-conjugating enzyme Ubc12                                                                      | UBE2M         |
| 182 | + | 2.366 | 2.189 | P19338        | Nucleolin                                                                                           | NCL           |
| 183 | + | 4.251 | 2.188 | P00374        | Dihydrofolate reductase                                                                             | DHFR          |
| 184 | + | 4.149 | 2.174 | P60900        | Proteasome subunit alpha type-6                                                                     | PSMA6         |
| 185 | + | 2.394 | 2.168 | P14868        | Aspartate--tRNA ligase, cytoplasmic                                                                 | DARS          |
| 186 | + | 3.430 | 2.168 | PODMV9;PODMV8 | Heat shock 70 kDa protein 1B;Heat shock 70 kDa protein 1A                                           | HSPA1B;HSPA1A |
| 187 | + | 2.338 | 2.167 | P38919        | Eukaryotic initiation factor 4A-III;Eukaryotic initiation factor 4A-III, N-terminally processed     | EIF4A3        |
| 188 | + | 5.988 | 2.162 | P08238        | Heat shock protein HSP 90-beta                                                                      | HSP90AB1      |
| 189 | + | 6.260 | 2.145 | P61221        | ATP-binding cassette sub-family E member 1                                                          | ABCE1         |
| 190 | + | 4.356 | 2.135 | Q9UKK9        | ADP-sugar pyrophosphatase                                                                           | NUDT5         |
| 191 | + | 2.683 | 2.121 | P62906        | 60S ribosomal protein L10a                                                                          | RPL10A        |
| 192 | + | 6.158 | 2.120 | Q8NCW5        | NAD(P)H-hydrate epimerase                                                                           | APOA1BP       |
| 193 | + | 4.202 | 2.102 | P04179        | Superoxide dismutase [Mn], mitochondrial                                                            | SOD2          |
| 194 | + | 3.578 | 2.089 | Q99497        | Protein deglycase DJ-1                                                                              | PARK7         |
| 195 | + | 4.961 | 2.077 | P23921        | Ribonucleoside-diphosphate reductase large subunit                                                  | RRM1          |
| 196 | + | 3.726 | 2.076 | P46782        | 40S ribosomal protein S5;40S ribosomal protein S5, N-terminally processed                           | RPS5          |
| 197 | + | 2.114 | 2.055 | P55010        | Eukaryotic translation initiation factor 5                                                          | EIF5          |
| 198 | + | 5.100 | 2.025 | Q15393        | Splicing factor 3B subunit 3                                                                        | SF3B3         |
| 199 | + | 3.647 | 1.980 | P17174        | Aspartate aminotransferase, cytoplasmic                                                             | GOT1          |
| 200 | + | 6.099 | 1.967 | Q15008        | 26S proteasome non-ATPase regulatory subunit 6                                                      | PSMD6         |
| 201 | + | 1.581 | 1.957 | P11940        | Polyadenylate-binding protein 1                                                                     | PABPC1        |

## Identified Proteins

|     |   |       |       |               |                                                                   |         |
|-----|---|-------|-------|---------------|-------------------------------------------------------------------|---------|
| 202 | + | 2.646 | 1.948 | P25789        | Proteasome subunit alpha type-4                                   | PSMA4   |
| 203 | + | 2.488 | 1.944 | P06737        | Glycogen phosphorylase, liver form                                | PYGL    |
| 204 | + | 2.562 | 1.940 | Q13151        | Heterogeneous nuclear ribonucleoprotein A0                        | HNRNPA0 |
| 205 | + | 2.467 | 1.930 | Q13347        | Eukaryotic translation initiation factor 3 subunit I              | EIF3I   |
| 206 | + | 4.605 | 1.925 | P30084        | Enoyl-CoA hydratase, mitochondrial                                | ECHS1   |
| 207 | + | 3.222 | 1.924 | P49736        | DNA replication licensing factor MCM2                             | MCM2    |
| 208 | + | 5.289 | 1.918 | Q9NZI8        | Insulin-like growth factor 2 mRNA-binding protein 1               | IGF2BP1 |
| 209 | + | 3.347 | 1.911 | P48739        | Phosphatidylinositol transfer protein beta isoform                | PITPNB  |
| 210 | + | 8.817 | 1.898 | P13639        | Elongation factor 2                                               | EEF2    |
| 211 | + | 1.614 | 1.893 | P12956        | X-ray repair cross-complementing protein 6                        | XRCC6   |
| 212 | + | 3.291 | 1.889 | Q14257        | Reticulocalbin-2                                                  | RCN2    |
| 213 | + | 1.756 | 1.865 | O15371        | Eukaryotic translation initiation factor 3 subunit D              | EIF3D   |
| 214 | + | 4.813 | 1.863 | P35998        | 26S protease regulatory subunit 7                                 | PSMC2   |
| 215 | + | 2.688 | 1.856 | P21333        | Filamin-A                                                         | FLNA    |
| 216 | + | 5.609 | 1.845 | O43242        | 26S proteasome non-ATPase regulatory subunit 3                    | PSMD3   |
| 217 | + | 2.965 | 1.837 | O43143        | Pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15         | DHX15   |
| 218 | + | 2.921 | 1.836 | P49321        | Nuclear autoantigenic sperm protein                               | NASP    |
| 219 | + | 3.575 | 1.831 | Q04917        | 14-3-3 protein eta                                                | YWHAH   |
| 220 | + | 3.426 | 1.810 | Q99798        | Aconitate hydratase, mitochondrial                                | ACO2    |
| 221 | + | 2.694 | 1.802 | P15311        | Ezrin                                                             | EZR     |
| 222 | + | 3.536 | 1.799 | O14929        | Histone acetyltransferase type B catalytic subunit                | HAT1    |
| 223 | + | 1.869 | 1.790 | O43809        | Cleavage and polyadenylation specificity factor subunit 5         | NUDT21  |
| 224 | + | 5.334 | 1.789 | P30041        | Peroxisredoxin-6                                                  | PRDX6   |
| 225 | + | 5.328 | 1.786 | P61163        | Alpha-centractin                                                  | ACTR1A  |
| 226 | + | 4.028 | 1.777 | P15170        | Eukaryotic peptide chain release factor GTP-binding subunit ERF3A | GSPT1   |
| 227 | + | 2.831 | 1.765 | P0DN79;P35520 | Cystathionine beta-synthase                                       | CBS     |
| 228 | + | 3.307 | 1.763 | P15121        | Aldose reductase                                                  | AKR1B1  |
| 229 | + | 4.117 | 1.762 | O43681        | ATPase ASNA1                                                      | ASNA1   |
| 230 | + | 5.276 | 1.751 | P49419        | Alpha-aminoadipic semialdehyde dehydrogenase                      | ALDH7A1 |

## Appendix

|     |   |       |       |               |                                                                                                                                                    |               |
|-----|---|-------|-------|---------------|----------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
| 231 | + | 3.028 | 1.746 | P31350        | Ribonucleoside-diphosphate reductase subunit M2                                                                                                    | RRM2          |
| 232 | + | 4.284 | 1.740 | Q9UHX1        | Poly(U)-binding-splicing factor PUF60                                                                                                              | PUF60         |
| 233 | + | 2.222 | 1.720 | O15067        | Phosphoribosylformylglycinamide synthase                                                                                                           | PFAS          |
| 234 | + | 2.007 | 1.709 | Q9BWD1        | Acetyl-CoA acetyltransferase, cytosolic                                                                                                            | ACAT2         |
| 235 | + | 4.327 | 1.702 | P24752        | Acetyl-CoA acetyltransferase, mitochondrial                                                                                                        | ACAT1         |
| 236 | + | 3.676 | 1.698 | P48643        | T-complex protein 1 subunit epsilon                                                                                                                | CCT5          |
| 237 | + | 2.765 | 1.697 | P33991        | DNA replication licensing factor MCM4                                                                                                              | MCM4          |
| 238 | + | 3.788 | 1.693 | P00491        | Purine nucleoside phosphorylase                                                                                                                    | PNP           |
| 239 | + | 1.738 | 1.684 | Q9UKY7        | Protein CDV3 homolog                                                                                                                               | CDV3          |
| 240 | + | 3.141 | 1.678 | P24666        | Low molecular weight phosphotyrosine protein phosphatase                                                                                           | ACP1          |
| 241 | + | 2.176 | 1.652 | O15355        | Protein phosphatase 1G                                                                                                                             | PPM1G         |
| 242 | + | 3.014 | 1.650 | P59998        | Actin-related protein 2/3 complex subunit 4                                                                                                        | ARPC4         |
| 243 | + | 2.024 | 1.633 | Q12874        | Splicing factor 3A subunit 3                                                                                                                       | SF3A3         |
| 244 | + | 2.585 | 1.621 | Q9BTT0        | Acidic leucine-rich nuclear phosphoprotein 32 family member E                                                                                      | ANP32E        |
| 245 | + | 3.336 | 1.605 | P60228        | Eukaryotic translation initiation factor 3 subunit E                                                                                               | EIF3E         |
| 246 | + | 2.167 | 1.598 | P38159;Q96E39 | RNA-binding motif protein, X chromosome;RNA-binding motif protein, X chromosome, N-terminally processed;RNA binding motif protein, X-linked-like-1 | RBMX;RBMXL1   |
| 247 | + | 2.723 | 1.558 | Q96AE4        | Far upstream element-binding protein 1                                                                                                             | FUBP1         |
| 248 | + | 1.628 | 1.549 | Q06203        | Amidophosphoribosyltransferase                                                                                                                     | PPAT          |
| 249 | + | 3.671 | 1.547 | P60842        | Eukaryotic initiation factor 4A-I                                                                                                                  | EIF4A1        |
| 250 | + | 1.876 | 1.538 | P51991        | Heterogeneous nuclear ribonucleoprotein A3                                                                                                         | HNRNPA3       |
| 251 | + | 1.573 | 1.537 | P27824        | Calnexin                                                                                                                                           | CANX          |
| 252 | + | 2.959 | 1.527 | P11310        | Medium-chain specific acyl-CoA dehydrogenase, mitochondrial                                                                                        | ACADM         |
| 253 | + | 1.291 | 1.523 | P63241;Q6IS14 | Eukaryotic translation initiation factor 5A-1;Eukaryotic translation initiation factor 5A-1-like                                                   | EIF5A;EIF5AL1 |
| 254 | + | 1.925 | 1.510 | P62241        | 40S ribosomal protein S8                                                                                                                           | RPS8          |
| 255 | + | 1.445 | 1.504 | O14744        | Protein arginine N-methyltransferase 5;Protein arginine N-methyltransferase 5, N-terminally processed                                              | PRMT5         |
| 256 | + | 2.996 | 1.488 | O14818;Q8TAA3 | Proteasome subunit alpha type-7;Proteasome subunit alpha type-7-like                                                                               | PSMA7;PSMA8   |
| 257 | + | 2.141 | 1.472 | Q13126        | S-methyl-5-thioadenosine phosphorylase                                                                                                             | MTAP          |
| 258 | + | 2.500 | 1.466 | P00492        | Hypoxanthine-guanine phosphoribosyltransferase                                                                                                     | HPRT1         |

## Identified Proteins

|     |   |       |       |               |                                                                                                                                 |                |
|-----|---|-------|-------|---------------|---------------------------------------------------------------------------------------------------------------------------------|----------------|
| 259 | + | 5.556 | 1.457 | Q00839        | Heterogeneous nuclear ribonucleoprotein U                                                                                       | HNRNPU         |
| 260 | + | 1.665 | 1.452 | Q8NC51        | Plasminogen activator inhibitor 1 RNA-binding protein                                                                           | SERBP1         |
| 261 | + | 1.677 | 1.451 | P00367;P49448 | Glutamate dehydrogenase 1, mitochondrial;Glutamate dehydrogenase 2, mitochondrial                                               | GLUD1;GLUD2    |
| 262 | + | 1.233 | 1.445 | Q07666        | KH domain-containing, RNA-binding, signal transduction-associated protein 1                                                     | KHDRBS1        |
| 263 | + | 2.746 | 1.433 | P17812        | CTP synthase 1                                                                                                                  | CTPS1          |
| 264 | + | 1.867 | 1.423 | O75874        | Isocitrate dehydrogenase [NADP] cytoplasmic                                                                                     | IDH1           |
| 265 | + | 3.928 | 1.414 | Q9NP79        | Vacuolar protein sorting-associated protein VTA1 homolog                                                                        | VTA1           |
| 266 | + | 2.799 | 1.412 | P52292        | Importin subunit alpha-1                                                                                                        | KPNA2          |
| 267 | + | 1.520 | 1.411 | Q14247        | Src substrate cortactin                                                                                                         | CTTN           |
| 268 | + | 2.818 | 1.409 | Q9NRN7        | L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase                                                       | AASDHPPT       |
| 269 | + | 2.450 | 1.408 | P61353        | 60S ribosomal protein L27                                                                                                       | RPL27          |
| 270 | + | 3.107 | 1.398 | Q9NZL9        | Methionine adenosyltransferase 2 subunit beta                                                                                   | MAT2B          |
| 271 | + | 1.732 | 1.384 | P11413        | Glucose-6-phosphate 1-dehydrogenase                                                                                             | G6PD           |
| 272 | + | 1.578 | 1.383 | Q9C0C9        | E2/E3 hybrid ubiquitin-protein ligase UBE2O                                                                                     | UBE2O          |
| 273 | + | 2.889 | 1.383 | P62851        | 40S ribosomal protein S25                                                                                                       | RPS25          |
| 274 | + | 4.228 | 1.380 | P61088;Q5JXB2 | Ubiquitin-conjugating enzyme E2 N;Putative ubiquitin-conjugating enzyme E2 N-like                                               | UBE2N;UBE2NL   |
| 275 | + | 2.277 | 1.374 | P32969        | 60S ribosomal protein L9                                                                                                        | RPL9           |
| 276 | + | 2.267 | 1.361 | Q13011        | Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial                                                                      | ECH1           |
| 277 | + | 2.706 | 1.355 | P62333        | 26S protease regulatory subunit 10B                                                                                             | PSMC6          |
| 278 | + | 4.773 | 1.353 | Q9Y2Z0        | Suppressor of G2 allele of SKP1 homolog                                                                                         | SUGT1          |
| 279 | + | 4.023 | 1.349 | P61978        | Heterogeneous nuclear ribonucleoprotein K                                                                                       | HNRNPK         |
| 280 | + | 2.559 | 1.311 | P30520        | Adenylosuccinate synthetase isozyme 2                                                                                           | ADSS           |
| 281 | + | 2.706 | 1.271 | Q15029        | 116 kDa U5 small nuclear ribonucleoprotein component                                                                            | EFTUD2         |
| 282 | + | 4.515 | 1.254 | O43175        | D-3-phosphoglycerate dehydrogenase                                                                                              | PHGDH          |
| 283 | + | 3.117 | 1.253 | P41091;Q2VIR3 | Eukaryotic translation initiation factor 2 subunit 3;Putative eukaryotic translation initiation factor 2 subunit 3-like protein | EIF2S3;EIF2S3L |
| 284 | + | 4.295 | 1.227 | Q9Y295        | Developmentally-regulated GTP-binding protein 1                                                                                 | DRG1           |

2.4 Proteins Significantly Enriched in ABPP with Probe C in Comparison to the Heat and Beads Controls

Table 10: Proteins significantly enriched in ABPP with probe C in comparison to the heat and beads control.

|   | Significant | −log p | Difference | Majority protein IDs | Protein names                             | Gene names |
|---|-------------|--------|------------|----------------------|-------------------------------------------|------------|
| 1 | +           | 8.840  | 4.380      | Q9Y490               | Talin-1                                   | TLN1       |
| 2 | +           | 7.145  | 4.369      | P35579               | Myosin-9                                  | MYH9       |
| 3 | +           | 5.252  | 3.489      | P21333               | Filamin-A                                 | FLNA       |
| 4 | +           | 3.407  | 3.390      | Q58FF8               | Putative heat shock protein HSP 90-beta 2 | HSP90AB2P  |
| 5 | +           | 4.635  | 3.314      | P39748               | Flap endonuclease 1                       | FEN1       |
| 6 | +           | 6.347  | 3.271      | P46940               | Ras GTPase-activating-like protein IQGAP1 | IQGAP1     |
| 7 | +           | 5.229  | 2.905      | P18206               | Vinculin                                  | VCL        |
| 8 | +           | 10.367 | 2.852      | P35580               | Myosin-10                                 | MYH10      |

2.5 Proteins Enriched in ABPP with Probe C that are Absent in the Competition Experiment

Table 11: Proteins significantly enriched in ABPP with probe C that are absent in the competition experiment.

|    | Significant | −log p | Difference | Majority protein IDs | Protein names                                                  | Gene names |
|----|-------------|--------|------------|----------------------|----------------------------------------------------------------|------------|
| 1  | +           | 5.807  | 2.613      | P12081               | Histidine--tRNA ligase, cytoplasmic                            | HARS       |
| 2  | +           | 4.009  | 2.612      | P12004               | Proliferating cell nuclear antigen                             | PCNA       |
| 3  | +           | 4.468  | 2.413      | P30084               | Enoyl-CoA hydratase, mitochondrial                             | ECHS1      |
| 4  | +           | 5.122  | 2.386      | Q9BXJ9               | N-alpha-acetyltransferase 15, NatA auxiliary subunit           | NAA15      |
| 5  | +           | 2.585  | 2.311      | Q15233               | Non-POU domain-containing octamer-binding protein              | NONO       |
| 6  | +           | 2.618  | 2.280      | Q9NR30               | Nucleolar RNA helicase 2                                       | DDX21      |
| 7  | +           | 4.819  | 2.241      | P62333               | 26S protease regulatory subunit 10B                            | PSMC6      |
| 8  | +           | 4.200  | 2.156      | Q9Y230               | RuvB-like 2                                                    | RUVBL2     |
| 9  | +           | 2.604  | 2.153      | P33316               | Deoxyuridine 5-triphosphate nucleotidohydrolase, mitochondrial | DUT        |
| 10 | +           | 4.357  | 2.035      | Q7L2H7               | Eukaryotic translation initiation factor 3 subunit M           | EIF3M      |

## Identified Proteins

|    |   |       |       |               |                                                                                |         |
|----|---|-------|-------|---------------|--------------------------------------------------------------------------------|---------|
| 11 | + | 5.332 | 1.958 | P0DN79;P35520 | Cystathionine beta-synthase                                                    | CBS     |
| 12 | + | 2.051 | 1.925 | P61247        | 40S ribosomal protein S3a                                                      | RPS3A   |
| 13 | + | 5.881 | 1.890 | P63261        | Actin, cytoplasmic 2;Actin, cytoplasmic 2, N-terminally processed              | ACTG1   |
| 14 | + | 3.581 | 1.877 | P35998        | 26S protease regulatory subunit 7                                              | P5MC2   |
| 15 | + | 2.713 | 1.861 | P11413        | Glucose-6-phosphate 1-dehydrogenase                                            | G6PD    |
| 16 | + | 3.284 | 1.845 | P08559        | Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial | PDHA1   |
| 17 | + | 3.467 | 1.811 | Q8N1G4        | Leucine-rich repeat-containing protein 47                                      | LRRC47  |
| 18 | + | 1.763 | 1.770 | Q13283        | Ras GTPase-activating protein-binding protein 1                                | G3BP1   |
| 19 | + | 1.393 | 1.757 | P00492        | Hypoxanthine-guanine phosphoribosyltransferase                                 | HPRT1   |
| 20 | + | 4.414 | 1.743 | Q15691        | Microtubule-associated protein RP/EB family member 1                           | MAPRE1  |
| 21 | + | 1.602 | 1.723 | O75874        | Isocitrate dehydrogenase [NADP] cytoplasmic                                    | IDH1    |
| 22 | + | 1.389 | 1.707 | P62633        | Cellular nucleic acid-binding protein                                          | CNBP    |
| 23 | + | 3.119 | 1.678 | Q9Y4L1        | Hypoxia up-regulated protein 1                                                 | HYOU1   |
| 24 | + | 1.785 | 1.678 | P62913        | 60S ribosomal protein L11                                                      | RPL11   |
| 25 | + | 1.869 | 1.655 | Q15459        | Splicing factor 3A subunit 1                                                   | SF3A1   |
| 26 | + | 5.164 | 1.654 | Q98XP5        | Serrate RNA effector molecule homolog                                          | SRRT    |
| 27 | + | 6.749 | 1.645 | Q9Y5K5        | Ubiquitin carboxyl-terminal hydrolase isozyme L5                               | UCHL5   |
| 28 | + | 4.244 | 1.600 | Q15717        | ELAV-like protein 1                                                            | ELAVL1  |
| 29 | + | 2.772 | 1.599 | Q96P70        | Importin-9                                                                     | IPO9    |
| 30 | + | 5.172 | 1.598 | P46777        | 60S ribosomal protein L5                                                       | RPL5    |
| 31 | + | 2.666 | 1.582 | Q9Y450        | HBS1-like protein                                                              | HBS1L   |
| 32 | + | 2.479 | 1.574 | Q98PX3        | Condensin complex subunit 3                                                    | NCAPG   |
| 33 | + | 2.067 | 1.560 | Q14204        | Cytoplasmic dynein 1 heavy chain 1                                             | DYNC1H1 |
| 34 | + | 3.255 | 1.536 | O43681        | ATPase ASNA1                                                                   | ASNA1   |
| 35 | + | 2.236 | 1.524 | Q14683        | Structural maintenance of chromosomes protein 1A                               | SMC1A   |
| 36 | + | 2.976 | 1.486 | O95801        | Tetratricopeptide repeat protein 4                                             | TTC4    |
| 37 | + | 4.327 | 1.452 | O75153        | Clustered mitochondria protein homolog                                         | CLUH    |
| 38 | + | 4.862 | 1.445 | Q6PI48        | Aspartate--tRNA ligase, mitochondrial                                          | DARS2   |
| 39 | + | 4.774 | 1.441 | Q15008        | 26S proteasome non-ATPase regulatory subunit 6                                 | PSMD6   |

2.6 Proteins Enriched in ABPP with Probe C that are Absent in the Heat Control

Table 12: Proteins significantly enriched in ABPP with probe C that are absent in the heat control.

|    | Significant | −log p | Difference | Majority protein IDs | Protein names                                            | Gene names |
|----|-------------|--------|------------|----------------------|----------------------------------------------------------|------------|
| 1  | +           | 5.573  | 4.443      | Q9Y490               | Talin-1                                                  | TLN1       |
| 2  | +           | 5.547  | 4.011      | P35579               | Myosin-9                                                 | MYH9       |
| 3  | +           | 4.335  | 3.679      | Q58FF8               | Putative heat shock protein HSP 90-beta 2                | HSP90AB2P  |
| 4  | +           | 5.303  | 3.535      | P39748               | Flap endonuclease 1                                      | FEN1       |
| 5  | +           | 8.374  | 3.311      | P18206               | Vinculin                                                 | VCL        |
| 6  | +           | 4.227  | 3.153      | P46940               | Ras GTPase-activating-like protein IQGAP1                | IQGAP1     |
| 7  | +           | 4.510  | 3.078      | Q8WUM4               | Programmed cell death 6-interacting protein              | PDCD6IP    |
| 8  | +           | 6.554  | 3.040      | Q15785               | Mitochondrial import receptor subunit TOM34              | TOMM34     |
| 9  | +           | 6.053  | 2.968      | P41250               | Glycine-tRNA ligase                                      | GARS       |
| 10 | +           | 1.257  | 2.844      | P61604               | 10 kDa heat shock protein, mitochondrial                 | HSPE1      |
| 11 | +           | 3.483  | 2.830      | O75369               | Filamin-B                                                | FLNB       |
| 12 | +           | 4.904  | 2.811      | P42704               | Leucine-rich PPR motif-containing protein, mitochondrial | LRPPRC     |
| 13 | +           | 5.932  | 2.753      | Q13838               | Spliceosome RNA helicase DDX39B                          | DDX39B     |
| 14 | +           | 4.951  | 2.748      | P35580               | Myosin-10                                                | MYH10      |
| 15 | +           | 4.620  | 2.675      | P26196               | Probable ATP-dependent RNA helicase DDX6                 | DDX6       |
| 16 | +           | 3.590  | 2.664      | Q7KZF4               | Staphylococcal nuclease domain-containing protein 1      | SND1       |
| 17 | +           | 5.795  | 2.633      | Q9Y2L1               | Exosome complex exonuclease RRP44                        | DIS3       |
| 18 | +           | 2.660  | 2.631      | Q00341               | Vigilin                                                  | HDLBP      |
| 19 | +           | 5.807  | 2.613      | P12081               | Histidine-tRNA ligase, cytoplasmic                       | HARS       |
| 20 | +           | 5.726  | 2.552      | P36776               | Lon protease homolog, mitochondrial                      | LONP1      |
| 21 | +           | 4.366  | 2.538      | P26358               | DNA (cytosine-5)-methyltransferase 1                     | DNMT1      |
| 22 | +           | 3.450  | 2.513      | Q00610               | Claithrin heavy chain 1                                  | CLTC       |
| 23 | +           | 3.265  | 2.493      | O95373               | Importin-7                                               | IPO7       |
| 24 | +           | 1.982  | 2.422      | O75643               | U5 small nuclear ribonucleoprotein 200 kDa helicase      | SNRNP200   |

## Identified Proteins

|    |   |       |       |        |                                                                                                                           |         |
|----|---|-------|-------|--------|---------------------------------------------------------------------------------------------------------------------------|---------|
| 25 | + | 2.338 | 2.403 | P43246 | DNA mismatch repair protein Msh2                                                                                          | MSH2    |
| 26 | + | 1.748 | 2.391 | P23246 | Splicing factor, proline- and glutamine-rich                                                                              | SFPQ    |
| 27 | + | 5.122 | 2.386 | Q9BXJ9 | N-alpha-acetyltransferase 15, NatA auxiliary subunit                                                                      | NAA15   |
| 28 | + | 5.627 | 2.348 | Q08J23 | tRNA (cytosine(34)-C(5))-methyltransferase                                                                                | NSUN2   |
| 29 | + | 2.585 | 2.311 | Q15233 | Non-POU domain-containing octamer-binding protein                                                                         | NONO    |
| 30 | + | 2.618 | 2.280 | Q9NR30 | Nucleolar RNA helicase 2                                                                                                  | DDX21   |
| 31 | + | 4.053 | 2.206 | P20042 | Eukaryotic translation initiation factor 2 subunit 2                                                                      | EIF2S2  |
| 32 | + | 4.200 | 2.156 | Q9Y230 | RuvB-like 2                                                                                                               | RUVBL2  |
| 33 | + | 2.769 | 2.129 | Q9Y6E2 | Basic leucine zipper and W2 domain-containing protein 2                                                                   | BZW2    |
| 34 | + | 3.741 | 2.115 | O00148 | ATP-dependent RNA helicase DDX39A                                                                                         | DDX39A  |
| 35 | + | 3.795 | 2.112 | O60841 | Eukaryotic translation initiation factor 5B                                                                               | EIF5B   |
| 36 | + | 5.974 | 2.094 | Q5VYK3 | Proteasome-associated protein ECM29 homolog                                                                               | ECM29   |
| 37 | + | 4.357 | 2.035 | Q7L2H7 | Eukaryotic translation initiation factor 3 subunit M                                                                      | EIF3M   |
| 38 | + | 6.608 | 2.019 | P62191 | 26S protease regulatory subunit 4                                                                                         | PSMC1   |
| 39 | + | 2.371 | 1.963 | Q8TEX9 | Importin-4                                                                                                                | IPO4    |
| 40 | + | 4.742 | 1.934 | P33176 | Kinesin-1 heavy chain                                                                                                     | KIF5B   |
| 41 | + | 5.072 | 1.924 | Q14152 | Eukaryotic translation initiation factor 3 subunit A                                                                      | EIF3A   |
| 42 | + | 5.702 | 1.912 | Q13642 | Four and a half LIM domains protein 1                                                                                     | FHL1    |
| 43 | + | 2.331 | 1.901 | O75534 | Cold shock domain-containing protein E1                                                                                   | CSDE1   |
| 44 | + | 1.285 | 1.870 | P04181 | Ornithine aminotransferase, mitochondrial;Ornithine aminotransferase, hepatic form;Ornithine aminotransferase, renal form | OAT     |
| 45 | + | 3.188 | 1.849 | Q86VP6 | Cullin-associated NEDD8-dissociated protein 1                                                                             | CAND1   |
| 46 | + | 3.284 | 1.845 | P08559 | Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial                                            | PDHA1   |
| 47 | + | 4.426 | 1.826 | P43897 | Elongation factor Ts, mitochondrial                                                                                       | TSFM    |
| 48 | + | 1.772 | 1.822 | O75937 | DnaJ homolog subfamily C member 8                                                                                         | DNAJC8  |
| 49 | + | 1.940 | 1.815 | P31689 | DnaJ homolog subfamily A member 1                                                                                         | DNAJA1  |
| 50 | + | 3.467 | 1.811 | Q8N1G4 | Leucine-rich repeat-containing protein 47                                                                                 | LRRC47  |
| 51 | + | 2.929 | 1.794 | P31942 | Heterogeneous nuclear ribonucleoprotein H3                                                                                | HNRNPH3 |
| 52 | + | 2.956 | 1.778 | P11172 | Uridine 5-monophosphate synthase;Orotate phosphoribosyltransferase;Orotidine 5-phosphate decarboxylase                    | UMPS    |

## Appendix

|    |   |       |       |        |                                                                                                            |          |
|----|---|-------|-------|--------|------------------------------------------------------------------------------------------------------------|----------|
| 53 | + | 1.763 | 1.770 | Q13283 | Ras GTPase-activating protein-binding protein 1                                                            | G3BP1    |
| 54 | + | 4.064 | 1.743 | O00154 | Cytosolic acyl coenzyme A thioester hydrolase                                                              | ACOT7    |
| 55 | + | 1.389 | 1.707 | P62633 | Cellular nucleic acid-binding protein                                                                      | CNBP     |
| 56 | + | 2.721 | 1.697 | Q9NPD8 | Ubiquitin-conjugating enzyme E2 T                                                                          | UBE2T    |
| 57 | + | 1.414 | 1.692 | P50454 | Serpin H1                                                                                                  | SERPINH1 |
| 58 | + | 3.119 | 1.678 | Q9Y4L1 | Hypoxia up-regulated protein 1                                                                             | HYOU1    |
| 59 | + | 1.785 | 1.678 | P62913 | 60S ribosomal protein L11                                                                                  | RPL11    |
| 60 | + | 6.955 | 1.660 | P49327 | Fatty acid synthase:[Acyl-carrier-protein] S-acetyltransferase:[Acyl-carrier-protein] S-malonyltransferase | FASN     |
| 61 | + | 1.869 | 1.655 | Q15459 | Splicing factor 3A subunit 1                                                                               | SF3A1    |
| 62 | + | 5.164 | 1.654 | Q9BXP5 | Serrate RNA effector molecule homolog                                                                      | SRRT     |
| 63 | + | 6.749 | 1.645 | Q9Y5K5 | Ubiquitin carboxyl-terminal hydrolase isozyme L5                                                           | UCHL5    |
| 64 | + | 2.807 | 1.623 | P45974 | Ubiquitin carboxyl-terminal hydrolase 5                                                                    | USP5     |
| 65 | + | 4.244 | 1.600 | Q15717 | ELAV-like protein 1                                                                                        | ELAVL1   |
| 66 | + | 2.772 | 1.599 | Q96P70 | Importin-9                                                                                                 | IPO9     |
| 67 | + | 2.666 | 1.582 | Q9Y450 | HBS1-like protein                                                                                          | HBS1L    |
| 68 | + | 3.743 | 1.581 | Q08211 | ATP-dependent RNA helicase A                                                                               | DHX9     |
| 69 | + | 2.479 | 1.574 | Q9BXP3 | Condensin complex subunit 3                                                                                | NCAPG    |
| 70 | + | 2.067 | 1.560 | Q14204 | Cytoplasmic dynein 1 heavy chain 1                                                                         | DYNC1H1  |
| 71 | + | 2.200 | 1.540 | Q9NUU7 | ATP-dependent RNA helicase DDX19A                                                                          | DDX19A   |
| 72 | + | 2.236 | 1.524 | Q14683 | Structural maintenance of chromosomes protein 1A                                                           | SMC1A    |
| 73 | + | 5.132 | 1.523 | Q13263 | Transcription intermediary factor 1-beta                                                                   | TRIM28   |
| 74 | + | 2.976 | 1.486 | O95801 | Tetratricopeptide repeat protein 4                                                                         | TTC4     |
| 75 | + | 4.327 | 1.452 | O75153 | Clustered mitochondria protein homolog                                                                     | CLUH     |
| 76 | + | 4.862 | 1.445 | Q6PI48 | Aspartate--tRNA ligase, mitochondrial                                                                      | DARS2    |
| 77 | + | 4.074 | 1.399 | Q7Z6Z7 | E3 ubiquitin-protein ligase HUWE1                                                                          | HUWE1    |

2.7 Proteins Enriched in the ABPP with Probe C that are Absent in the Heat and Competition Control

Table 13: Proteins significantly enriched in ABPP with probe C that are absent in the heat and competition control.

|    | Significant | −log p | Difference | Majority protein IDs | Protein names                                                                  | Gene names |
|----|-------------|--------|------------|----------------------|--------------------------------------------------------------------------------|------------|
| 1  | +           | 5.807  | 2.613      | P12081               | Histidine–tRNA ligase, cytoplasmic                                             | HARS       |
| 2  | +           | 5.122  | 2.386      | Q9BXJ9               | N-alpha-acetyltransferase 15, NatA auxiliary subunit                           | NAA15      |
| 3  | +           | 2.585  | 2.311      | Q15233               | Non-POU domain-containing octamer-binding protein                              | NONO       |
| 4  | +           | 2.618  | 2.280      | Q9NR30               | Nucleolar RNA helicase 2                                                       | DDX21      |
| 5  | +           | 4.200  | 2.156      | Q9Y230               | RuvB-like 2                                                                    | RUVBL2     |
| 6  | +           | 4.357  | 2.035      | Q7L2H7               | Eukaryotic translation initiation factor 3 subunit M                           | EIF3M      |
| 7  | +           | 3.284  | 1.845      | P08559               | Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial | PDHA1      |
| 8  | +           | 3.467  | 1.811      | Q8N1G4               | Leucine-rich repeat-containing protein 47                                      | LRRC47     |
| 9  | +           | 1.763  | 1.770      | Q13283               | Ras GTPase-activating protein-binding protein 1                                | G3BP1      |
| 10 | +           | 1.389  | 1.707      | P62633               | Cellular nucleic acid-binding protein                                          | CNBP       |
| 11 | +           | 3.119  | 1.678      | Q9Y4L1               | Hypoxia up-regulated protein 1                                                 | HYOU1      |
| 12 | +           | 1.785  | 1.678      | P62913               | 60S ribosomal protein L11                                                      | RPL11      |
| 13 | +           | 1.869  | 1.655      | Q15459               | Splicing factor 3A subunit 1                                                   | SF3A1      |
| 14 | +           | 5.164  | 1.654      | Q9BXP5               | Serrate RNA effector molecule homolog                                          | SRRT       |
| 15 | +           | 6.749  | 1.645      | Q9Y5K5               | Ubiquitin carboxyl-terminal hydrolase isozyme L5                               | UCHL5      |
| 16 | +           | 4.244  | 1.600      | Q15717               | ELAV-like protein 1                                                            | ELAVL1     |
| 17 | +           | 2.772  | 1.599      | Q96P70               | Importin-9                                                                     | IPO9       |
| 18 | +           | 2.666  | 1.582      | Q9Y450               | HBS1-like protein                                                              | HBS1L      |
| 19 | +           | 2.479  | 1.574      | Q9BPX3               | Condensin complex subunit 3                                                    | NCAPG      |
| 20 | +           | 2.067  | 1.560      | Q14204               | Cytoplasmic dynein 1 heavy chain 1                                             | DYNC1H1    |
| 21 | +           | 2.236  | 1.524      | Q14683               | Structural maintenance of chromosomes protein 1A                               | SMC1A      |
| 22 | +           | 2.976  | 1.486      | O95801               | Tetratricopeptide repeat protein 4                                             | TTC4       |
| 23 | +           | 4.327  | 1.452      | O75153               | Clustered mitochondria protein homolog                                         | CLUH       |
| 24 | +           | 4.862  | 1.445      | Q6PI48               | Aspartate–tRNA ligase, mitochondrial                                           | DARS2      |

### 3 Compounds Identified as Potential Hit from the High-Throughput Screening

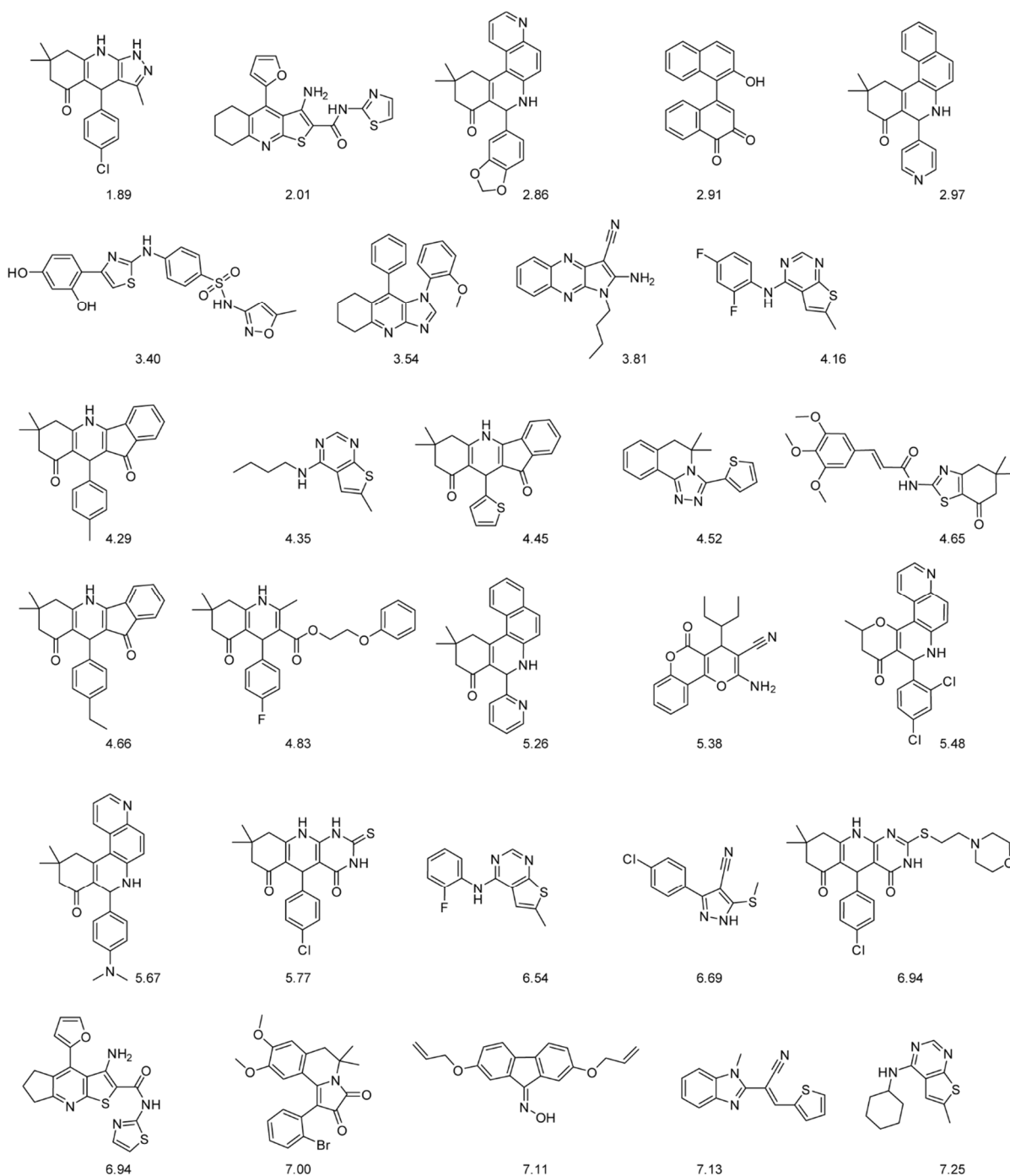


**Figure 59:** Hit compounds identified in high-throughput screening. 29 of 45 compounds identified as potential Fhit inhibitors. Numbers indicate the residual Fhit activity in %.

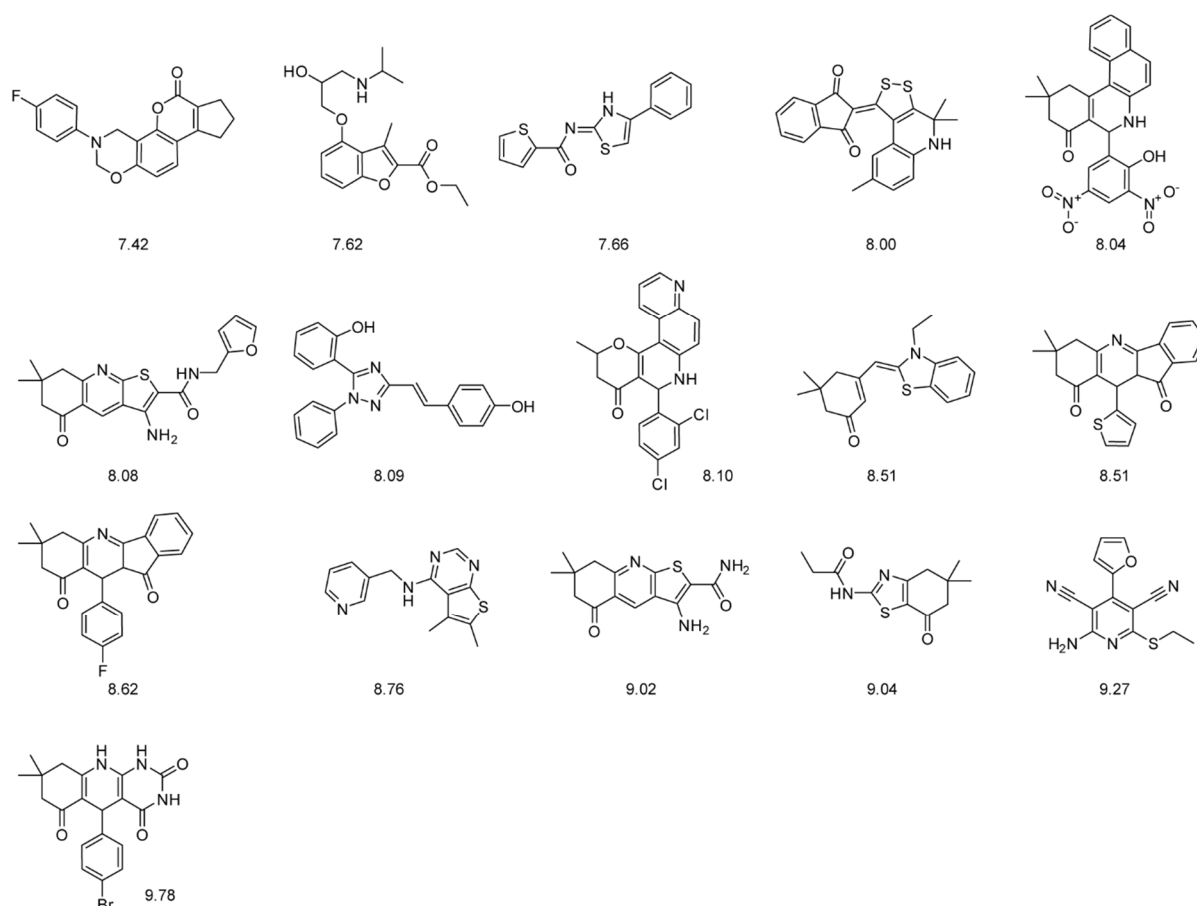


**Figure 60:** Hit compounds identified in high-throughput screening 2. 16 of 45 compounds identified as potential Fhit inhibitors. Numbers indicate the residual Fhit activity in %.

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